STUDIES ON MALARIA IN SERRA DO NAVIO REGION, AMAPA STATE, BRAZIL.

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This work is dedicated to my husband Pedro, to my daughters Flávia and Patrícia and to my parents.

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ABSTRACT

This study evaluated the status of malaria transmission in Serra do Navio region where a malaria control programme had been carried out for more than 20 years, which included the provision of a daily intake of chloroquinised salt.

Malaria endemicity in the study areas [target area: Serra do Navio (SNV); control areas: Colonia Agua Branca (CAB), Porto Terezinha (PT) and Arrependido (ARR)] was established based on the spleen rate and prevalence of malaria parasites among the 2-9 years age group. SNV was defined as a non-endemic area for malaria since both rates were zero, while CAB and ARR were concluded to be mesoendemic areas since the rates were between 10 and 50%, and PT was considered hypoendemic area because both rates were under 10%.

Antibody prevalence measured using IFAT with asexual form antigens of *Plasmodium falciparum* and *Plasmodium vivax* and ELISA (asexual forms of *P.falciparum*, sporozoites of *P.falciparum, P.vivax type 1, P.vivax type 2, P.vivax type 3* and *P.malariae/P.brasilianum*) was determined for individuals from each study area. These results showed a high frequency of *P.falciparum* antibodies in all areas.

The level of haptoglobin in 100 children from all these areas demonstrated the close relationship between hypohaptoglobinaemia and malaria in the control areas.

Haskin's method , a qualitative method, was applied for testing chloroquine in urine. Therefore, chloroquine levels were measured in serum and urine using the ELISA test and the results were in agreement with the reports of the intake of chloroquine salt.

The distribution and potentiality of the malaria vectors in

all areas was determined. Fifteen anopheline species were identified among 3053 mosquitoes collected by human biting catches in the 4 study areas. 96.4% of the total mosquitoes caught belonged to 4 species, namely, *An.albitarsis, An.braziliensis, An.nuñeztovari* and *An.triannulatus*. *An.darlingi* which is considered the main vector of malaria in the Amazon region of Brazil was very scarce. Using ELISA for the detection of *Plasmodium spp* sporozoites a positivity rate of 0.799% (23/2876) was found covering six species: 15 *An. albitarsis*, 4 *An. nuñeztovari*, and 1 of each: *An. braziliensis, An. triannulatus, An. oswaldoi* and *An.rangeli*. 9 out 23 positive mosquitoes were infected with *P. malariae* ; 9 with *P. vivax*- variant VK 247; 3 with *P. falciparum* and 2 with the classical *P.vivax*.

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ABBREVIATIONS

AA.....amino acids
Ab.....antibody
AMQ.....amodiaquine
AMQR.....amodiaquine resistance
AMQ-R....amodiaquine-resistant
AMQ-S....amodiaquine-sensitive
AP.....Amapá State
API.....annual parasite incidence
ARR.....Arrependido
ART.....artemisinin
bp.....base pair(s)
BR.....Brazil
BSA.....bovine serum albumin
CAB.....Colonia Agua Branca
CEM.....programme of malaria eradication
CHS.....chloroquinised salt
CIPC.....chloroquine-induced pigment clumping
CM.....culture medium
C.O.....cut off
CQ.....chloroquine
CQR.....chloroquine resistance
CQ-R.....chloroquine - resistant
CQ-S.....chloroquine - sensitive
CS.....circumsporozoite
CSP.....circumsporozoite protein
CYG .....cycloguanil
dH2O.....distilled water
DHF .....dihydrofolate
DHFR ....dihydrofolate reductase
DHPS.....dihydropteroate synthase
DNA.....deoxyribonucleic acid
dNTPs....deoxyribonucleotides
EBV.....Epstein-Barr virus
EDTA ....ethylenediaminetetraacetic acid
EE .....exo-erythrocytic

ELISA....enzyme-linked immunoassay
FITC.....fluorescein isothiocyanate
FNS......Fundação Nacional de Saúde
FP.......ferriprotoporphyrin
FUNAI....Fundação Nacional de Ajuda ao Indio
GDH......glutamic dehydrogenase
G-6-PD...glucose-6-phosphate
Hb.......haemoglobin
HbF......fetal haemoglobin
HC.......haematocrit
HK.......Haskin's method
HMS......hyper-reactive malarial splenomegaly syndrome
IC.......inibitory concentration
ICOMI....Indústria e Comércio de Mineração S.A.
ID.......identification
IDH......isocitric dehydrogenase
IDR......immunodominant region
IEC......Instituto Evandro Chagas
IFAT.....immunofluorescent antibody test
IgG......immunoglobulin G
IMP......inosine monophosphate
LDH......lactate dehydrogenase
M........molar
MDR......multidrug resistance
mdr......multidrug resistance gene
mer......merozoite
MH.......malaria history
MHC......major histocompatibility complex
MIC......minimum inibitory concentration
MoAb.....monoclonal antibody
MQ.......mefloquine
MQR......mefloquine resistance
MQ-R.....mefloquine-resistant
MQ-S.....mefloquine-sensitive
MW.......molecular weight(s)
NAD......nicotinamide adenine dinucleotide
NADP.....NAD phosphate

NaHCO3...sodium hydrogen carbonate
NaOH.....sodium hydroxide
NAPRT....nicotinic acid phosphoribosyltransferase
NP-40....Nonidet P-40
NPRT.....nicotinamide phosphoribosyltransferase
NK.......natural killer cells
OD.......optical density
OPD......ortho-phenilenediamine????
PABA.....p-amino benzoic acid
PAHO.....Pan American Health Organization
PBS......phosphate buffered saline
PBS-T-M..phosphate buffered saline-Tween 20-milk
PCR......polymerase chain reaction
(%)p.....(percent) parasitemia
PE.......phosphatidylethanolamine
PER......peroxidase
PF.......*Plasmodium falciparum*
pf*mdr*....***Plasmodium falciparum*** multidrug resistance gene
PF.......***Plasmodium falciparum***
PG.......proguanil
PNG......Papua New Guinea
PQ.......primaquine
PS.......phosphatidylserine
PT.......Porto Terezinha
PV.......*Plasmodium vivax*
PVM......parasitophorous vacuolar membrane
PYR......pyrimethamine
PYRR.....pyrimethamine resistance
PYR-R....pyrimethamine-resistant
PYR-S....pyrimethamine-sensitive
QBC......quantitative buffy coat
QUI......quinine
QUIR.....quinine resistance
QUI-R....quinine resistant
QUI-S....quinine sensitive
rbc(s)...red blood cell(s)
RFLP.....restriction fragment length polymorphism

RID.....radial immunodiffusion
RNA.....ribonucleic acid
rRNA.....ribosomal RNA
RT.....room temperature
SD.....standard deviation
SL.....positive slide
SN.....supernatant
SNV.....Serra do Navio
SOD.....superoxide dismutase
SR.....spleen rate
SSC.....saline sodium citrate
SUCAM....Superintendência de Campanhas
THF.....tetrahydrofolate
TNF.....tumour necrosis factor
TSS.....Tropical splenomegaly syndrome
UMP.....uridine monophosphate
UV.....ultraviolet
v/v.....volume for volume
w/v.....weight for volume
WHO.....World Health Organization

CHAPTER 1: Introduction

1.1 Malaria parasites

Malaria infection in man is caused by protozoa that belong to the **Family Plasmodiidae** within the **Order Coccidiidae.** The genus, *Plasmodium,* is characterized by exo-erythrocytic schizogony, that is an asexual multiplication by division occurring in the parenchymal cells of the liver of the vertebrate host, and *a further schizogony in the erythrocytes.* The four species of malaria parasites of man are: *Plasmodium malariae* (Laveran, 1881); *Plasmodium vivax* (Grassi and Feletti, 1890); *Plasmodium falciparum* Welch, 1897; and *Plasmodium ovale* Stephen, 1922. *P. falciparum* is the most lethal.

1.2 Life cycle

The human malaria parasites have, basically, the same life cycle. The cycle has two phases: sporogony (exogenous sexual phase) - with multiplication in *Anopheles* mosquitoes; and schizogony (endogenous asexual phase) with multiplication in the vertebrate host. The schizogony takes place in the red blood cells (erythrocytic schizogony) and in the parenchyma cells of the liver (exo-erythrocytic schizogony). In exo-erythrocytic schizogony, there is a primary or pre-erythrocytic schizogony that follows the development of the sporozoite and the delayed exo-erythrocytic schizogony which is related to relapses in *Plasmodium vivax* and *Plasmodium ovale* infections (Fig 1.2). The infection in man is initiated through the bite of an infected female anopheline mosquito that inoculates sporozoites into the blood stream. Sporozoites, then go to the liver and invade hepatocytes where the exo-erythrocytic schizogony takes place (asexual division). The infected hepatocytes burst, releasing merozoites into the blood stream which invade the erythrocytes and begin the erythrocytic schizogony. In this phase the merozoite develops from the ring form through the trophozoite to the

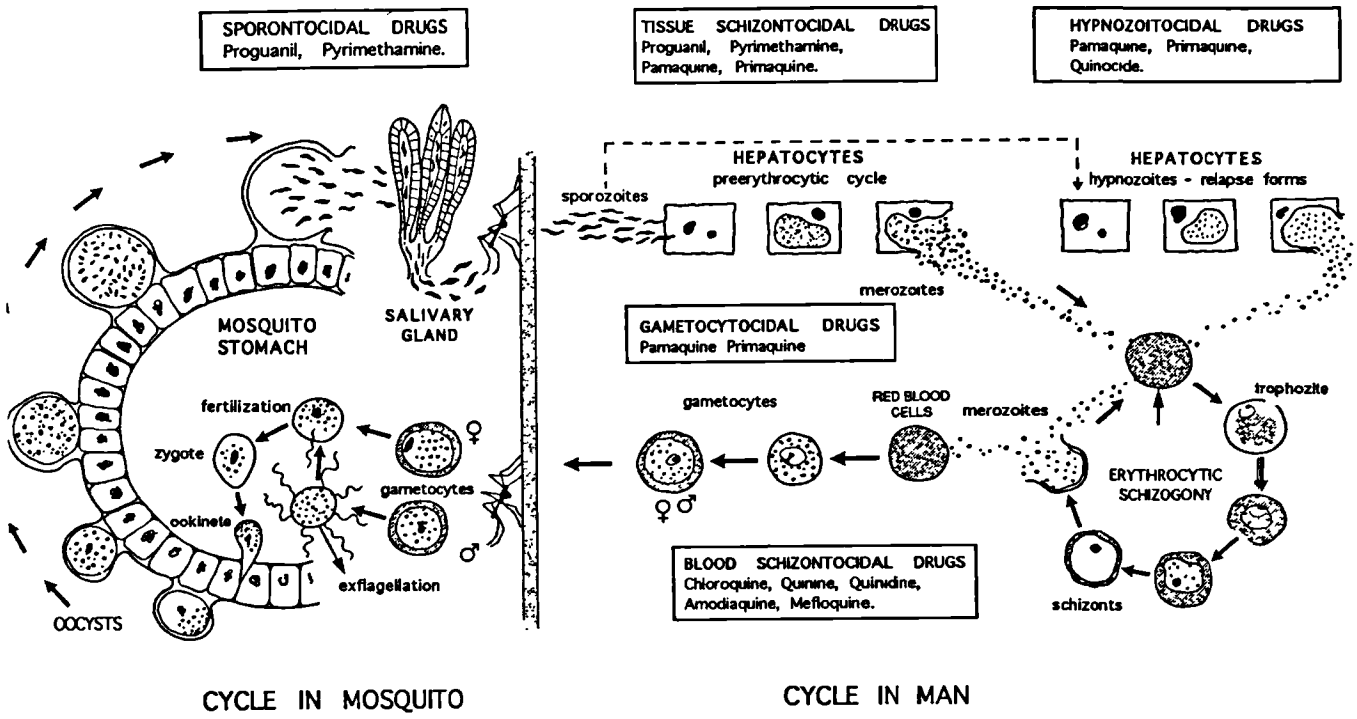


Figure 1.2

Life cycle of malaria parasites

(produced by Payne and supplied by Dr. Warhurst)

mature schizont, containing 8-24 daughter merozoites which, after the erythrocyte ruptures, will initiate a subsequent intraerythrocytic cycle.
After a period of asexual replication, some infected merozoites differentiate into female and male gametocytes. These forms, which are infective to the anopheline mosquito during blood feeding, mate in the mosquitos' midgut and produce zygotes that develop into ookinetes and then invade the gut wall to form oocysts. Sporogony follows and as a result thousands of sporozoites are produced, which are released into the mosquito's haemocoel and invade the salivary glands to await transmission to the vertebrate host.

1.3 Morphology

The morphological differences among the four species of human malaria parasites are shown in table 1.3 (Warhurst & Williams, 1989). The differentiation is based on forms seen on Giemsa-stained thin films of peripheral blood.
Plasmodium brasilianum, a monkey malaria parasite, was discovered in a cacajao monkey (*Calcajao calvus*), from the State of Amazonas in Brazil by Gonder *et al.*, 1908 (*vide* Garnham, 1966). Evidence has been found supporting its morphological and genetical similarity to *Plasmodium malariae* of man (Garnham, 1966 and Lal *et al.*, 1988). Even its quartian periodicity in erythrocytic schizogony is the same as in *P. malariae*.

1.4 Clinical features of malaria infections

The clinical response to infection depends on the species of *Plasmodium* and on the immunological status of the patient.
The incubation period in malaria starts when an infected mosquito inoculates sporozoites into the bloodstream of the host, and ends when the first clinical signs appear, of which fever is the most common. This period usually lasts from 9 to 30 days and its length is correlated with the

TABLE 1.3

MORPHOLOGICAL DIFFERENCES BETWEEN *Plasmodium* SPECIES OF MAN.

	SPECIES			
	P.FALCIPARUM	***P.VIVAX***	***P.MALARIAE***	***P.OVALE***
Trophozoites a)ring forms	0.15-0.5 of diameter of RBC which is normal in size. young rings: cytoplasm very fine; old rings: cytoplasm thick irregular. Marginal (accolé) and 2 chromatin dots forms and multiple infections common.	0.3-0.5 of diameter of RBC which is unaltered in size. Cytoplasm: circle, thin.	0.3-0.5 of diameter of RBC which is unaltered in size. Cytoplasm: circle, thick.	0.3 diameter of RBC which is unaltered in size. Cytoplasm: circle, thicker.
Growing forms	RBC unaltered, sometimes stippled, pale. Parasite compact; Pigment dense brown or black mass.	RBC enlarged, stippled. Parasite: amoeboid, vacuolate; Pigment fine and scattered, golden brown	RBC unaltered. Parasite compact, rounded or band-shape: Pigment: dark brown or black, often concentrated in a line along one edge of band.	RBC unaltered in size, or slightly enlarged; may be oval & fimbriated. Parasite: compact, rounded; Pigment fine brown grains.
Mature schizont	RBC unaltered, sometimes stippled, pale. Parasites about 0.6 of RBC; Nuclei or merozoites: 8-24; Pigment clumped, black.	RBC much enlarged, stippled. Parasite large, filling enlarged RBC; Nuclei or merozoites: 12-24; Pigrent: a golden brown central loose mass.	RBC unaltered. Parasite fills RBC completely; Nuclei or merozoites: 6-12, usually 8, sometimes forming rosette; Pigment: brown black central clump.	RBC frequently oval, fimbriated, enlarged, stippled. Parasite as for *P.malariae* but does not entirely fill the slightly enlarged RBC; Pigment; brown central clump
Gametocytes	RBC distorted. parasite crescentic (banana shape).	RBC enlarged, stippled. Parasite large, rounded, filling enlarged RBC.	RBC unaltered. Parasite small, round, filling RBC.	RBC slightly enlarged, stippled. Parasite round.
Stippling	Maurer's clefts.	Schuffner's dots.	None.	Jame's dots.

RBC - red blood cell

species and strain of the parasite, with the intensity of the infection, with the previous treatment, with the degree of resistance of the host, and with previous exposure to malaria.

The prepatent period in malaria lasts from the time of infection to the first finding of malaria parasites in the blood. This period also is correlated with the species of *Plasmodium* since the rate of growth of the preerythrocytic stage and number of merozoites produced is variable among the species.

The patent period in malaria refers to the time when clinical manifestations coincide with detectable parasitaemia in samples of peripheral blood.

1.4.1 *Plasmodium falciparum*

The incubation period of *P.falciparum* is variable and lasts from 7 to 14 days.

Acute infection by falciparum malaria causes prolonged and irregular high fever, intense headache, pains in the back and legs, anorexia, prostration, nausea, vomiting and occasional diarrhoea. Where the parasitaemia is successfully controlled by the immune response, parasitaemia may persist for 6 months, or at most for 18 months.

Severe infection that is related to high parasitaemia, commonly gives rise to hyperpyrexia, convulsions,stupor, collapse, copious vomiting, diarrhoea, haemolytic anaemia and jaundice.

In holoendemic and hyperendemic areas for falciparum malaria, young children over the age of six months are in the group in which most severe malaria occurs. Probably, older children and adults are not so vulnerable due to the acquisition of partial immunity.

Both children and adults can have severe malaria when they live in areas of low endemicity for falciparum malaria (Warrell *et al.*, 1990).

Cerebral malaria, hypoglycaemia, pulmonary oedema, acute

renal failure and massive haemolysis are the result of the complications of falciparum malaria.
Splenomegaly and progressive anaemia are clinical signs of chronic or repeated infections.

1.4.2 *Plasmodium vivax*

Vivax malaria has a period of incubation that varies between 12 and 17 days, but it may last from 8 to 11 months.
P.vivax infection is characterized by its long-term development, intermittent fever with paroxysms on alternate days, and splenomegaly. Relapse (renewed occurrence of clinical symptoms and/or parasitaemia after a time considerably greater than the intervals between periodic paroxysms of the primary infection, Bruce-Chwatt, 1985) may occur after a variable period of latency.
The primary attack starts with headache, fever, pain in the back, prostration and nausea. This symptoms are milder or absent in relapses or in patients who have had previous infections.

1.4.3 *Plasmodium malariae*

The incubation period in malaria caused by *P.malariae* is not shorter than 18 days and can be longer, such as 40 days.
The clinical signs of this infection resemble that of vivax malaria, but are less severe that the latter. The paroxysm of *P.malariae* infection is regular (every 72 hours) and usually occurs in the late afternoon. The anaemia is mild, when present; splenomegaly is frequent. The infection develops slowly and gradually.
Recrudescences in *P.malariae* are most frequent during the first year, but the blood remains infective and recrudescence may occur at long intervals (up to 52 years) after the last exposure to infection (Bruce-Chwatt, 1985).

1.4.4 *Plasmodium brasilianum*

Although this is a simian malaria parasite infective to man, the transmission cycles of human and simian malaria are independent (Deane, 1969).

The infectivity of this species to man was demonstrated experimentally by Contacos & Coatney, (1963) when they used *A. freeborni* heavily infected with sporozoites of *P. brasilianum* for biting man. The period of incubation varied from 24 to 64 days. Parasitaemia was always low (250 parasites per mm^3).

Clinical signs were mainly the quartan periodicity as in *P. malariae* infection, and splenomegaly was observed in some infections.

1.5 Pathogenesis

The pathophysiological mechanisms that govern the clinical manifestations caused by malaria are not completely understood, although it is well known that those manifestations result directly or indirectly from asexual stage parasites. The remarkable feature of malaria is fever, but anaemia, thrombocytopenia, splenomegaly and water and electrolyte disturbance are also present in malaria caused by all four human malaria parasites. Complications such as severe anaemia, renal and pulmonary failure, and cerebral dysfunction can occur in falciparum malaria. Nephrotic syndrome is related to chronic infection with *P. malariae*.

Malaria has also been implicated as a cofactor in the pathogenesis of African Burkitt's lymphoma and of tropical splenomegaly syndrome (TSS) or hyper-reactive malarial splenomegaly syndrome (HMS) (Bryceson *et al.*, 1983).

Burkitt's lymphoma is mainly found in tropical areas where malaria is highly prevalent. There are two hypothesis for the role of malaria in its pathology: 1- malaria increases B cell turnover promoting chromosomal translocation, thus causing the transfer of the *c-myc* oncogene (in chromosome 8) to an area where it permits unrestrained growth of B

cells (Klein, 1979) and; 2- malaria impairs the host's ability to control the growth of EBV- infected immortalized B cells once those have been formed (Greenwood, 1987)
In TSS the role of malaria in the aetiology is not clear, since even though the patients develop massive splenomegaly and a very high level of antimalarial and non-specific IgM, they do not have detectable parasitaemias and some cases do not respond to antimalarial chemotherapy. However, there is strong evidence that malaria has a dominant role, such as: 1- the HMS is found only in areas where malaria is endemic; 2- possession of the haemoglobin genotype AS provides protection against HMS; 3- individuals with HMS have higher levels of malaria AB than controls and; 4- level of gammaglobulin and splenomegaly regress in many patients who take malaria chemoprophylaxis (Greenwood & Whittle, 1981). Repeated malaria infections stimulate further immunoglobulin production and, serum levels of IgM and autoantibodies increase during life in residents of malarious areas (Greenwood, 1987).

1.5.1 Fever

Although a correlation between schizont rupture and onset of fever has been established and explains the periodicity pattern of fever in synchronized infection, the mechanisms of fever remain unclear. No exogenous pyrogen released from infected cells has been identified. However, the possibility that the ingestion of parasite debris released at the time of schizont rupture may trigger release of an endogenous pyrogen (specifically, interleukin 1) from Kupffer cells and other macrophages has been proposed (see section 1.6.3).

1.5.2 Anaemia

It is known that the red cell destruction caused by parasitization with resulting haemolysis, does not entirely account for the anaemia, since the degree of anaemia exceeds the parasite density.

P.falciparum can cause a severe, acute haemolytic anaemia and in endemic areas the young children with this severe condition have only a low parasite rate which, generally, is preceded by recent and acute febrile illness. Thus, their anaemia may be a result of previous severe and untreated *P. falciparum* infection (Greenwood, 1987).
Mechanisms such as disturbed function of the bone marrow and the increase of rbc turnover caused by repeated malaria episodes which can lead to deficiencies in rbc production, have been suggested as explanations for how low parasitaemia could cause anaemia.
The role of immuno factors in the pathogenesis of malaria anaemia has been point out by Greenwood & Whittle, (1981), who showed that sera from residents in malarious areas contained heterophilic antibodies, cold agglutinins and antibodies that reacted with altered human rbc, although there was no proof of their haemolytic action *in vivo*.
An alternative explanation is that the nonimmunological changes in uninfected erythrocytes (alterations in lipid content of erythrocyte membranes) make these cells more susceptible to splenic removal. Also, opsonization of uninfected cells by antibody may take place (Sherman,1991).

1.5.3 Sequestration

In falciparum malaria the phenomenon of cytoadherence is basic to the locally diminished tissue perfusion seen in its more severe complications (see section 1.5).
Cytoadherence is the result of the expression on the surface of the rbc containing later trophozoite or schizonts of strain- and stage-specific parasite and, probably host cell-derived ligands, which adhere to 2 specific receptors on the endothelial cells. Ockenhouse & Chulay, (1988) identified some of the ligands, and White & Dance, (1988) reported that the glycoprotein thrombospondin may be involved in the specific receptor complex. Thus, the small vessels become plugged by masses of parasitized rbc and the consequent ischaemia produces symptoms that vary

intense malaria transmission, acquire slowly and gradually a partially protective immunity through constant boosting by living parasites and protection is manifested by lower peak parasitaemias and a relative tolerance to clinical complications of infection (McGregor & Giles, 1960). However, in *P.vivax* infection, the individual acquires a potent immunity to an homologous strain of the parasite but not to an heterologous one (Boyd, 1942). Thus, the effective host immunity is strain-specific.

Experiments have demonstrated that immunoglobulins only reduce the asexual parasitaemia and do not affect the density of circulating gametocytes. This fact has focused attention on the role of specific antibodies in mediating antiplasmodial defence and has also established that this humoral defence is stage-specific. The concept of antibody-dependent defence in malaria infection has been the main impetus for investigators who are trying to identify target (vaccine) antigens.

Studies on immunity to sporozoites in humans have shown that older children and adults residing in areas where malaria is endemic develop antibodies to sporozoites which are not detectable in younger children. Although there is no evidence that these antibodies provide protection, the belief that a sporozoite vaccine could be effective still motivates further research (Titus et al., 1991).

Merozoite invasion of the erythrocyte stimulates the production of antibodies, some of which will prevent invasion. However, these invasion-blocking antibodies do not uniformly correlate with the immune status. Merozoite antigens have been isolated and they are targets for vaccine production (see section 1.6.5).

The maturation of malaria parasites after entering erythrocytes can be stopped by antibody and other soluble factors. It is believed that antibodies binding to the surface of infected erythrocytes might interfere with the parasite's intracellular development and could also exert a protective effect by facilitating removal of the cells

from the circulation. The latter can be promoted by splenic macrophages and Kupffer cells and may depend on antibody-facilitated phagocytosis. Based on simian malaria studies, the antibody that binds to appropriate antigens on *P. falciparum*-infected erythrocytes can also interfere with cytoadherence to endothelial cells. This antibody excludes the mature forms from the sites of sequestration and permits their removal from circulation by the spleen that is capable of filtering out of circulation those plasmodia-infected cells. Target antigens in this defence have been considered as potential vaccine candidates.
A role for complement in defence against malaria infection has not been established (Titus et al., 1991).

1.6.3 Cell mediated immunity

Basic cellular immunology has made remarkable progress during recent years which has provided a better understanding of cellular effector mechanisms. As a result, it is now possible to consider that infected erythrocytes might be targets of cytotoxic cells, including natural killer (NK) cells or antibody-dependent or -independent cytotoxic T lymphocytes. The NK can be involved in innate immunity while the latter can be involved in acquired immunity. The activated macrophage, theoretically, can also be involved in malaria defence, perhaps by elaboration of toxic oxygen radicals or soluble factors such as monokines. Other T-cell products such as gamma-interferon can activate the macrophages to exert antiparasitic effects.
Activated macrophages, as in almost all inflammatory processes, induce the production of cytokines such as TNF, IL-1 and IL-6. They are products of different genes that encode non-homologous proteins and bind distinct receptors. However, their cellular source and their biological activities overlap. TNF and IL-1 can induce self biosynthesis, biosynthesis of each other and of IL-6. Therefore, all these three cytokines often act synergistically (Titus *et al.*, 1991).

with the organ involved and the degree of tissue anoxia. In cerebral malaria, the cerebral and other vessels contain infected erythrocytes, but in the brain the proportion of parasitized rbc is much higher than in other organs due to the sequestration (MacPherson *et al.*, 1985). This cytoadherence phenomenon is probably an adaptation to allow the rbc containing later stage parasites to avoid passage through the spleen and consequent destruction.

Evidence suggests that the mechanism of malaria pathogenesis is more a result of proteins (cytokines) secreted by the host's cells in response to the presence of the parasite than due to the direct effect of the parasite on the rbc. Tumour necrosis factor (TNF), a protein also known as cachectin, and interleukins are involved in the host response to infection (see section 1.6.3), since a direct relationship between elevated TNF levels and mortality from cerebral malaria was found by Grau *et al.*, (1989). Grau, (1992) stated that in cerebral malaria, pathogenesis appear to be a result of a cytokine cascade mediated by the immune response and that TNF might be involved in the pathogenesis of cerebral malaria.

The pathogenesis of nephrotic syndrome is probably a result of immune complex deposition, since deposits of IgG, IgM and C3 in the glomeruli have been reported and electron-dense deposits have been observed in the mesangium.

Chronic *P. malariae* infection can cause full-blown nephrotic syndrome in children as a result of membranoproliferative glomerulonephritis, which can evolve into total glomerulosclerosis. The aetiological role played by malaria in this syndrome is still in doubt because antimalarial chemotherapy is not effective, although immune complexes containing *P. malariae* antigens have been identified in the glomeruli (Allison *et al.*, 1969 and Ward & Kibukamusoke, 1969).

1.6 Immunology of malaria

1.6.1 Innate immunity

Newborns are innately resistant to acute infection and it is uncommon to become ill with malaria during the first 3 months of life, and when so, the manifestation is mild with low parasitaemia. The mechanisms responsible for this resistance to malaria at birth and early life is not completely known. This resistance may be due to passive immunity acquired from immune mothers and lasts for a few months (Edozien *et al.*, 1962).

Nonimmunological factors such as erythrocytes containing large amounts of fetal haemoglobin (HbF) which was demonstrated to retard the growth of parasites (Pasvol *et al.*, 1976, 1977), and the age of erythrocytes since old rbc containing HbF do not support *P. falciparum* (Wilson *et al.*, 1977; Pasvol *et al.*, 1980) must be taken in consideration. Also, there are some indications that diet (PABA or iron deficiency), and abnormal haemoglobins (such as sickle haemoglobin) may mediate innate immunity (Wyler, 1990).

Gilles *et al.*, (1967) has shown that children with sickle cell trait (heterozygous haemoglobin - AS) in malarious areas have lower parasitaemia than children with normal haemoglobin (AA). In addition, Colbourne & Edington, (1956) and Edington, (1967) demonstrated that fatal malaria is more frequent among normal children than those who are heterozygous. It was observed by Adeloye *et al.*, (1971) and Luzzatto, (1974) that homozygous children [sickle cell anaemia (SS)] were not protected against malaria since malaria can be fatal within this group.

Thalassaemias are believed to protect against malaria, since HbF persists in thalassaemic infants (Pasvol *et al.*, 1977) and parasitized thalassaemic erythrocytes bind antimalarial IgG with more avidity than those from non-thalassaemic ones. Thus, this fact may result in more efficient splenic clearance of parasitized cells in thalassaemia (Warrell *et al.*, 1990). On the other hand, HbE and beta-thalassaemia in Thailand do not confer changes in

parasite rate, parasite density or mortality of falciparum malaria in children (Kruatrachue *et al.*, 1969).

Glucose-6-phosphate (G-6-PD) deficiency as a protection against malaria remains under discussion since some investigators have found a significant correlation between this deficiency and lower parasitaemia and others have not (Allison & Clyde, 1961; Gilles *et al.*, 1967 and Martin *et al.*, 1979). However, Bienzle *et al.*, (1972) found potential protection against malaria in heterozygous females with intermediate enzyme activity. Beside that, Luzzatto, (1979) has demonstrated that when both G-6-PD normal and G-6-PD deficient rbc are available in the circulation, *P. falciparum* infects preferentially the normal cells. The mechanism of oxidant stress acting synergistically with G-6-PD deficiency to confer resistance to malaria infection has been suggested by Huheey & Martin, (1975) and Golenser *et al.*, (1983).

A distinctive type of hereditary ovalocytosis has been found among Papua New Guinea and some areas of the South-east Asian population (Amato & Booth, 1977). The ovalocytic erythrocytes were resistant to infection by *P.falciparum* in culture suggesting their role in this innate resistance to malaria infection (Kidson *et al.*, 1981).

1.6.2 Humoral Immunity

Antibody (Ab) to erythrocytic asexual stages becomes detectable a few days after the blood is invaded by the parasite. High level Ab is found within the next two weeks and can persist longer than the parasitological crisis. Ab lasts for a variable period of time and depends on the duration of infection.

In relapses or reinfection the Ab rises more rapidly and reaches higher levels than in the initial infection (Collins *et al.*, 1964 a, b, c; Lupascu *et al.*, 1966).

It is known that malaria infection only confers a partial protective immunity in man. Individuals living in endemic areas where they are exposed to repeated infections and

Many effects of cytokines could enable the host to resist the disease, but their overproduction can increase the pathology as seen in cerebral malaria where TNF may be largely responsible for pathology (Clark *et al.*, 1987 and 1989, Grau *et al.*, 1989 and 1992). TNF as well as IL1 may also be responsible for the fever that follows schizont rupture in patients with malaria (Titus *et al.*, 1991).
Despite the contradictory reports available in the literature, IL-1 may also play an important role in malaria immunity. As a clue, Curfs *et al.*, (1990) demonstrated that treatment with IL-1 prevents cerebral malaria and reduces parasitaemia in mice infected with *P. berghei*.
The role of IL-6 in the pathogenesis of malaria is still not understood. Kern *et al.*, (1989) and Grau *et al.*, (1989) reported that the serum levels of IL-6 increase in mice with *P. berghei* and in patients with *P. falciparum*, respectively. However, Grau *et al.*, (1990) have suggested that IL-6 is not involved in the pathogenesis of cerebral malaria since they failed to prevent cerebral malaria in mice infected with *P. berghei* by treating them with an anti-IL-6 antibody.

1.6.4 Malaria in Pregnancy

During pregnancy, acquired immunity to malaria can be completely suppressed, which makes the pregnant woman vulnerable to severe falciparum malaria. For unexplained reasons, the primiparae are more susceptible. Semi immune pregnant women, do not generally have the acute febrile illness that is observed in children, but can develop anaemia which in some cases may be severe anaemia (Fleming, 1981). As demonstrated by McGregor *et al.*, (1983), low level infections in pregnant women can cause parasitisation of the placenta, which is important since lowering of the birth weight can occur with a poor prognosis
The loss of immunity remains unexplained. Increased levels of steroidal hormones are, however, believed to play a role in the increased susceptibility of pregnant women to

certain nonparasitic infectious diseases and this may well be involved (Wyler, 1990).

1.6.5 Vaccines

The types of vaccines that have been under investigation are proteins from the sporozoite, asexual blood stage and from the gamete, zygote or ookinete (transmission blocking).

The development of sporozoite vaccines is based on the target antigen of antisporozoite immunity which appears to be the major surface coat protein of mature sporozoites (circumsporozoite protein - CS). This protein contains repetitive epitopes corresponding to oligopeptides tandemly repeated and flanked by homologous sequences in all *Plasmodium* species. From species to species, the number of repeats, the composition and size of the oligopeptides that establish the repetitive epitope, are variable.

Recombinant CS antigen produced from different *Plasmodium* species and synthetic peptides of various lengths coupled to carrier proteins can be tested as potential vaccines. There are already indications of problems, however, with all possible vaccines, since they apparently elicit low antibody titers that are inconsistently boosted by repeated inoculation (Wyler, 1990).

The vaccines using asexual parasite proteins from merozoites and intraeythrocytic forms have as target those antigens expressed on the surface of the merozoites, those associated with surface membranes of infected erythrocytes and rhoptry protein (table 1.6.5). Although they are antigenically more complex and diverse than the CS, the motivation to develop vaccines using these antigens is based on two facts, 1- these stages are the targets of defence in natural infections, and 2- since pathology is associated only with the blood stages, morbidity and mortality can be dramatically reduced even if only partial protection can be achieved (Wyler, 1990).

The transmission-blocking vaccines are based on the

possibility of inducing production of antibody against sexual stages. These antibodies, which would act in the blood meal of the mosquito, might interfere in the development of the sporogonic cycle.

The immunodominant target epitopes are expressed on macrogametes and are lost soon after fertilization. There are also other antigens on the zygote and ookinete that are targets for this type of vaccine (table 1.6.5).

The antigens shown in table 1.6.5 are the present targets for vaccines, but they do not represent all of the potential candidates since new antigens for both asexual blood and sexual stages of *P. falciparum* and *P. vivax* are rapidly being discovered (Mendis, 1991).

Therefore, those antigens already used in human trials, such as the sporozoite vaccine and asexual blood stage vaccine, have raised questions about the immune response to malaria antigens and the degree of protection they can afford in a human malaria vaccine, respectively (Mendis, 1991).

The advantage of using vaccine antigens expressed only in the mosquito is that they are not exposed to the immune system and are therefore less likely to have variable epitopes (Ya-Ping *et al.*, 1992).

1.7 Diagnosis

1.7.1 Microscopy of blood films

Despite great advances in malariology since Laveran's discovery of the malaria parasite in 1881, microscopy remains the best method of diagnosis. It is a simple, relatively inexpensive diagnostic technique that is based on the morphological features showed in section 1.3. Microscopy is sensitive; a well trained microscopist can detect a very low parasitaemia such as one parasite per microliter of blood (Dowling & Shute, 1966). From examination of thick and thin blood film the species, stage, and intensity of infection can be determined. However, this technique has limitations since microscopy is

TABLE 1.6.5

POTENTIAL MALARIA VACCINE CANDIDATES

TARGET ANTIGENS	LOCATION	TARGET PARASITE SPECIES
$(NANP)_3$	sporozoite surface (CS- protein)	*P. falciparum*
1- PMMSA, P190, gp195, P185, PSA, MSP-1 and MSA-1 2- MSA-2 3- PV200 4- NH_2-terminal synthetic peptide of the glycoprotein 5- NH_2-terminal synthetic peptide of the glycoprotein used in a multiple vaccine 6- rhoptry antigens	merozoite surface (glycoprotein) merozoite	1- *P. falciparum* 2- *P. falciparum* (Anders & Brown, 1990) 3- *P.vivax* (Del Portillo et *al.*, 1991) 4- *P. falciparum* 5- *P. falciparum* (Patarroyo et *al.*, 1988) 6- *P. falciparum* (Anders & Brown, 1990)
1- 230 Da protein, 48/45 Da protein 2- GAM-1	gamete	1- *P. falciparum* 2- *P. vivax*
Pfs25	zygote and ookinete	*P. falciparum*

time consuming and tedious; routine examination of a thick blood film takes 5-10 minutes or longer in the case of low-level parasitaemia (WHO, 1986). It requires trained personnel, a high-powered microscope and can be insensitive if prior chemoprophylaxis has been taken (Warhurst, 1990). Alternatively, there is the fluorescence microscopy by the QBC (quantitative buffy coat-commercial capillary tubes containing acridine orange dye). Its use as a method for replacing the conventional microscopy of Giemsa-stained thick blood films was tested and discussed by Baird *et al.*, (1992). They concluded that the lack of a public electricity power source, the sensitivity of the tubes to humidity, the failure to detect low-level parasitaemia and the cost of the test are the limitations of this method for diagnosis of malaria in field conditions.

1.7.2 Antibody detection

It is possible to detect the production of circulating antibodies by the host in response to the parasite infection. During the acute phase of malaria infection there is a low concentration of antibodies in the circulation which is a limitation for the application of such tests in the diagnosis of acute malaria. However, this is a very sensitive technique for measurement of the intensity of exposure to malaria in population.

1.7.2.1 Indirect immunofluorescence test (IFAT)

In the indirect fluorescent antibody test (IFAT) the whole parasite either from culture or infected blood is used as antigen. Short term or continuous cultured parasites of *P. falciparum* are the best source of antigen since mature trophozoites or schizonts give the most sensitive test. Fluorescein-labelled antihuman globulin is used to detect the antiparasite antibody on the surface of parasites in thick films.

This test detects antibody very soon after the blood is invaded but can give negative results with sera from

children. However, the number of false negative results in this test are lower than in other tests, probably due to the use of a crude antigen.

1.7.2.2 Enzyme-linked immunoassay (ELISA)

ELISA, the enzyme linked immunosorbent assay, is analogous to IFAT, except that the antigen is coated onto a microplate and the conjugate is an antiglobulin labelled with an enzyme, which is then quantitated by means of a chromogenic substrate.

This test had been widely applied to malaria with a wide variety of specific antigens. There is general agreement that there are more false negative results with this test than with IFAT, probably because the antigen may not be optimal.

1.7.3 Antigen detection

Detection of parasite antigen or nucleic acids can be used both in epidemiology and diagnosis since current infection is measured.

The techniques can either use monoclonal or polyclonal antibodies for the detection of the parasites. ELISA can also be used for the detection of antigens and the principle and development of the technique is the same as for antibody detection, except that the plate is coated with a monoclonal or polyclonal antibody.

1.7.3.1 Monoclonal antibodies (MoAb)

The monoclonal antibodies are highly specific since they only react with their corresponding antigen.

A large range of MoAb that are stage- and species-specific for malaria parasites has been produced. They have been used for clinical diagnosis, in which the infected blood is tested against a range of MoAb by an IFAT. The antigenic diversity of the different stages of malaria parasite has, however, limited their application.

1.7.3.2 DNA hybridization (probes)

Nucleic acid probes are based on the principle that there are parasite-specific regions in the parasite DNA to which short, specific, labelled oligonucleotides will bind or hybridize. Most DNA probes used to detect *P. falciparum* are based on a repeated 21-base pair (bp) sequence of genomic DNA (Hyde, 1990). Studies using DNA probes have been able to detect small quantities of *P. falciparum* DNA (Barker *et al.*, 1989; Boonsaeng *et al*,. 1989; Franzen *et al.*, 1984; Holmberg *et al.*, 1987; McLaughlin *et al.*, 1987; Sethabutr *et al.*, 1988). DNA probes have also been used to detect *P. falciparum* in mosquitoes (Holmberg et al., 1987) and for species identification in the *Anopheles gambiae* complex (Gale & Crampton, 1987). The sensitivity of these probes varies, but they may detect as few as 5-10pg to 1ng DNA per sample, which is equivalent to 100 to 10,000 ring stage parasites (WHO, 1986; Goman et al. 1982). However in general they are less sensitive than skilled microscopy in detecting scanty parasitaemias. The advantage of DNA probes over microscopy is that many samples can be examined simultaneously. Thus DNA probes could be useful in large epidemiological studies where great numbers of blood samples are examined in a relatively short period of time, for screening blood in blood banks, or in identification and/or incrimination of *Anopheles* mosquitoes. A major limitation of DNA probes is labelling with radioactive isotopes. Although radioactive labelling confers a high degree of sensitivity to the probes, the danger associated with these short-life isotopes and the requirement for special handling and repeated relabelling limits their usage. Non-radioactive DNA probe techniques have been developed based on enzyme-linked colorimetric or chemiluminescent detection systems (McLaughlin *et al.*, 1987). Sethabutr *et al.*, (1988) have shown study that non-radioactive probes compare favourably with radioisotopes which was confirmed by Wilson *et al.*, (1992), while others report at least a 10-fold reduction in sensitivity (Hughes

et al., 1990). The sensitivity of probes is enhanced if the target sequence is highly abundant. DNA probes to the 21-bp repeat region take advantage of this principle. Recently, ribosomal RNA (rRNA) has been suggested as an appropriate target to increase sensitivity because there is 10-50 times more RNA than DNA in malaria trophozoites (of which 90-95% of the nucleic acid may be rRNA). Hence, probes to rRNA might theoretically be at least 100-fold more sensitive probes to DNA (Waters & McCutchan, 1990). However, the stability of the rRNA in the sample is a limitation for the use of its probe. Small ring stages, however, have a relatively small content of rRNA compared with later forms, so sensitivity and advantage over DNA-probes depend on the stage circulating.

1.8 Cultivation *in vitro*

Attempts to cultivate malaria parasites *in vitro* date from 1912, when limited multiplication of human plasmodia was achieved. Further attempts were made by many research workers until 1976 when an extremely simple method of *in vitro* cultivation was discovered for *Plasmodium falciparum* (Trager and Jensen, 1976). This method, with some modifications and improvements, has also allowed the cultivation of some plasmodia of monkeys (*P.knowlesi, P.cynomolgi, P.inui* and *P.fragile*) and partial success was obtained for the cultivation of the human plasmodia, *P.malariae* and *P.vivax* (Bruce-Chwatt, 1985).

1.9 Biochemistry and metabolism

1.9.1 Energy metabolism

The primary energy source for the intraerythrocytic stages of malaria parasites is glucose from the blood. These stages lack carbohydrate reserves. When compared with normal erythrocytes the infected red blood cells (rbcs) show O_2 uptake stimulated by glucose. However, *P. falciparum* does not oxidise glucose completely to carbon dioxide and water; instead it is a microaerophilic homolactate

fermenter, converting glucose mainly to lactate.
The enzymes of the Embden-Meyerhoff glycolytic pathway play a major role in conversion of glucose to lactate with the production of ATP, since all of them have been identified in extracts of *P. falciparum* (Roth *et al.*, 1988). *P. falciparum* also contains the non-glycolytic enzymes such as glutamic dehydrogenase (GDH), isocitric dehydrogenase (IDH), malic dehydrogenase and glutamic-oxaloacetic transaminase.
Deslauriers *et al.*, (1982) provided evidence that there are different ways for regulating glycolysis in host cell and parasite since the phosphofructokinase and lactic dehydrogenase of *Plasmodium* are less sensitive to high concentrations of substrate and low pH than those of the rbcs.

1.9.2 Synthetic processes

The hexose monophosphate shunt pathway allows the infected rbcs to produce ribose and NADPH which are important in nucleic acid synthesis and thus in nuclear division due to the requirement for purine, pyrimidine and ribose for nucleic acid synthesis.
The malaria parasite is sensitive to active forms of oxygen. However, it has been shown that *P. falciparum* and *P. berghei* do not synthesize their own superoxide dismutase (SOD) and so their lysosomes obtain SOD from host cells (Sherman, 1991).
Like other malaria parasites *P. falciparum* is capable of fixing carbon dioxide possibly producing isocitrate, although the role of this pathway remains unclear in the overall metabolism of the parasite.
Both, nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP) are involved in glycolysis and in the hexose monophosphate shunt pathway for ribose synthesis. Zerez *et al.*, (1990) had shown an increase of NAD content in *P. falciparum* infected cells. This increase could be due to synthesis from both nicotinamide and nicotinic acid

since levels of nicotinic acid phosphoribosyltransferase (NAPRT), nicotinamide phosphoribosyltransferase (NPRT) and nicotinamide deamidase were also increased in *P. falciparum* infected cells.

Most malarias demonstrated the glycolytic enzyme lactate dehydrogenase (LDH) and GDH activity. NADH can be oxidized via LDH, and NADPH via GDH or IDH (Vander Jagt *et al.*, 1989). NADPH is possibly formed from NADH via an ATP-requiring transhydrogenase.

The malaria parasite requires oxygen, but at a low level. Oxygen is the final acceptor for electrons from the cytochrome chain which have been transported via the ubiquinones. The availability of oxidised ubiquinone in the mitochondria allows the enzyme dihydroorotate dehydrogenase to catalyse the conversion of dihydroorotate to orotate in the *de novo* formation of pyrimidines (see later).

Spermine and spermidine, polyamines, are always found in biological materials and increased levels are found in rapidly growing cells. Both are involved in protein synthesis and contribute to ribosome and possibly membrane stability. They are synthesised from methionine and ornithine.

The growing *Plasmodium* requires increased amounts of material from the host cell and the extracellular environment. Consequently, an increase in the flux across the erythrocyte membrane may be essential for the survival of the intracellular parasite. Changes in the permeability of the membrane of the parasite-infected rbcs have been observed, but a clear biochemical description of the mechanism of such changes is not yet available (Sherman, 1991).

1.9.2.1 Pyrimidine synthesis

The mature mammalian erythrocyte loses its capacity to synthesise pyrimidines *de novo*. Intraerythrocytic *Plasmodium* contains a functional *de novo* pathway (Scheibel & Sherman, 1988). In extracts of *Plasmodium falciparum* 3

of the 5 enzymes (dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase) have been identified which catalyse the conversion of carbamylaspartate to UMP (uridine monophosphate).
Thymidylate synthetase, an enzyme converting uridylate to thymidilate, is also found in *Plasmodium* extracts, where it exists as bifunctional protein in combination with the enzyme of the pathway folate, dihydrofolate reductase (DHFR) which produces its essential co-factor, tetrahydrofolate (THF).

1.9.2.2 Purine salvage

Malaria parasites require a supply of preformed purines since there is no functional *de novo* purine biosynthetic pathway in these parasites.
Five enzymes (adenosine deaminase, adenosine kinase, adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase and purine nucleoside phosphorylase) the purine salvage pathway have been found in *P. falciparum* homogenates.
It has also been demonstrated that other species of *Plasmodium* contain all enzymes necessary for purine salvage (Sherman, 1991).

1.9.2.3 Nucleic acids

The amount of DNA contained in the malaria parasite increases 10-20 fold from merozoites to the mature forms after schizogonic development. In *P. falciparum* DNA synthesis begins at the early trophozoite and increases logarithmically until late schizogony.
The RNA of malaria parasites is mainly localized in cytoplasmic ribosomes (rRNA). The amount of RNA in the intraerythrocytic stage of malaria is about five times greater than the DNA (Sherman, 1991).
Shippen-Lentz *et al.*, (1990) demonstrated that malaria parasites possess at least five classes of rRNA

transcription units, and McCutchan *et al.*, (1988) has shown that only one of the two small subscript rRNA genes is expressed during erythrocytic development. The stability and turnover of plasmodial ribosomes and the manner of transcriptional regulation of the rRNA genes are unknown. The molecular mechanism responsible for plasmodial ribosomal protein synthesis in the cytoplasm is typically eukaryotic.

1.9.3 Nutrition

The nutrient uptake of the intraeythrocytic stages of *Plasmodium* can be effected by pinocytosis, diffusion and/or transport and bulk ingestion of host cell cytoplasm via the cytosome (specialized organelle).

The early stages of malaria parasites contain a tiny food vacuole bounded by a single membrane, and have small granules of malaria pigment, haemozoin. The haemozoin is a blackish brown pigment that is the end product of haemoglobin degradation. The chemical composition of haemozoin remains under discussion and was first believed to be melanin, later haematin or haematin coupled to a polypeptide. Afterwards, Yamada & Sherman (1979) demonstrated that the pigment of *P. lophurae* consists of insoluble monomers and dimers of haematin [ferriprotoporphyrin (FP)] coupled to a plasmodial protein, and methaemoglobin. For *P. falciparum*, Fitch & Kanjananggulpan, (1987) claimed that haemozoin was an aggregate of insoluble haematin, while Ashong *et al.*, (1989) claimed FP was coupled to a plasmodial protein.

Malaria parasites as well as their host cells are not able to synthesize fatty acids or cholesterol *de novo*, but they can form phosphatidylcholine and phosphatidyl ethanolamine (PE) via *de novo* pathways, or by methylation of PE or decarboxylation of phosphatidylserine (PS), respectively (Vial *et al.*, 1990).

The blood plasma serves as the primary source of the fatty acids required for plasmodial membrane formation.

1.9.4 Cell invasion

The sequence of the erythrocyte invasion by the malaria parasite is: recognition and attachment by the merozoite stage to specific receptors on the erythrocyte surface; junction formation between the merozoite apical region and the red cell membrane; discharge of the contents of the apical organelles (micronemes and rhoptries); invagination of the erythrocyte membrane to enclose the merozoite within a vacuole; and resealing of the rbc membrane after parasite entry is complete (Sherman, 1991).

The biochemistry of the recognition has been described for several *Plasmodium* species. Barnwell *et al.*, (1989); Wertheimer & Barnwell., (1989) confirmed the Duffy glycoprotein as the major receptor on human rbc for *P. knowlesi* and *P. vivax*. For *P. falciparum*, the sialic acid rich glycophorins appear to be the principal receptor.

There is no biochemical knowledge for the understanding of junction formation.

Rhoptries, micronemes and microspheres are the three membrane-bound organelles which are found at the apical end of the merozoite. It is believed that their chemical nature is lipidic and Mikkelsen *et al.*, (1988) claimed that the apical organelles phospholipids were involved in invasion and formation of the parasitophorous vacuolar membrane (PVM).

The invaginated PVM is depleted of IMP (inosine monophosphate). The PVM has not been isolated from any species of *Plasmodium* and the knowledge that we have about it, is that it enlarges with the intracellular growth of the parasite, it differs in its IMP composition and in its reactivity with cationized ferritin, and it is lacking in the rbc membrane proteins, spectrin, glycophorin band 3 and ankyrin (Sherman *et al.*, 1988; Dluzewski *et al.*, 1989).

1.10 Treatment

Cure of *P.falciparum* and *P.malariae* infections can be achieved through the use of blood schizontocides while for

P.vivax and *P.ovale* infections, a hypnozoitocide has to be added. Constraints on the treatment of malaria are mainly drug toxicity and the spread and development of drug resistance. In developing countries, in addition to difficulty in obtaining recommended drugs through the public health system, the indiscriminate use of available drugs by the general population encourages the development of resistance.

1.10.1 *Plasmodium falciparum*

Chloroquine is used for the treatment of the acute infection but only against those strains which are sensitive to this drug. Amodiaquine is therapeutically equivalent to chloroquine and cures some chloroquine resistant strains (WHO, 1990).

Quinine is used in the treatment of complicated malaria caused by *P.falciparum*, cerebral malaria, hyperpyrexia and/or for the cure of *P.falciparum* infections due to chloroquine resistant strains (Geary *et al*, 1986; WHO, 1990).

Looareesuwan *et al.*, (1990) have reported the loss of effectiveness of quinine in some endemic areas of malaria. Where quinine alone has no effect against *P.falciparum* malaria infection, either quinine + mefloquine or quinine + fansidar or quinine + tetracycline can be used (Mashaal, 1986).

Mefloquine may be used alone for the treatment of acute malaria infection due to multiple-drug resistant strains of *P.falciparum* (WHO, 1990).

Fansidar is used for the treatment of acute *P.falciparum* infection, but only for the susceptible strains and where chloroquine-resistant strains are known to occur.

Qinghaosu (artemisinin) and its derivatives have been used in China for the treatment of *P.falciparum* chloroquine resistant infections. Thaithong & Beale, (1985) had demonstrated the highly efficacy *in vitro* of artemisinin and artesunate on isolates of *P. falciparum* from Thailand,

independently of their susceptibility to chloroquine, pyrimethamine or pyrimethamine/sulfadoxine. The efficacy of artemisinin derivates was confirmed by Bunnag *et al.*, (1991) in a clinical trial carried out in adult males with acute uncomplicated and acute complicated falciparum malaria, where the cure rate was above 75%. On the other hand White *et al.*, (1992) in the Gambia compared the efficacy of i.m. artemether against i.m. chloroquine in children with moderate or severe falciparum malaria and demonstrated that artemether treatment enhanced parasite clearance in moderate malaria while in severe malaria there was difference between the two treatments.
Halofantrine has been used for treatment of acute malaria in adults from the Solomon Islands (Parkinson *et al*, 1989),in Pakistan (Rab *et al*, 1989), in children in Kenya (Watkins *et al*, 1989), in Malawi (Wirima *et al*, 1989), in children in Gabon (Richard-Lenoble *et al*, 1989), and has been shown to be an effective blood schizontocide.

1.10.2 *Plasmodium vivax*

Chloroquine remains the drug of choice for the treatment of naturally acquired *P.vivax* malaria aided by primaquine for the elimination of dormant tissue forms (hypnozoites). When the infection is acquired congenitally, from transfusions or from contaminated injections, primaquine is not required since the hypnozoite stages is not present. There have been some reports of chloroquine resistance.

1.10.3 *Plasmodium malariae*

Chloroquine is still the drug of choice for this species of *Plasmodium*. The other blood schizontocide drugs can also be used.

1.11 Drug resistance

The antimalarial drugs are classified biologically taking into account the varying degrees of susceptibility exhibited by the various developmental stages in the life

TABLE 1.11

CLASSIFICATION OF ANTIMALARIAL DRUGS ACCORDING TO THEIR BIOLOGICAL ACTIVITY

(based on Bruce-Chwatt *et al*, 1985)

CLASS	ACTIVITY ON PARASITES	EFFECTS
Tissue schizontocides	inhibition of growing EE stages in hepatocytes	prevent clinical malaria by stopping production and release of EE merozoites into the blood
Hypnozoitocides (Peters, 1983)	inhibition of latent EE stages (hypnozoites) in hepatocytes	radical cure of *P.v* and *P.o* infections by preventing relapse
Blood schizontocides	act on asexual blood stages of all human malaria parasites and on their gametocytes, except on mature *P.f* gametocytes	clinical cure of malaria or suppression of blood infection
Gametocytocides	active against the sexual forms of all human malaria parasites either in the human host or in the mosquito vector	disrupts transmission by preventing development of gametocytes in mosquitoes
Sporontocides	inhibit the development of oocysts and sporozoites in the vector	interrupts the cycle in mosquitoes, thus preventing transmission

P.f. = *P. falciparum*; P.v. = *P. vivax*; P.o. = *P. ovale*;
EE = exo-erythrocytic

cycle of malaria parasites as shown in the table 1.11. Drug resistance of *Plasmodium* species, mainly *P. falciparum* was first reported in 1910 when inadequate responses to high and regular doses of quinine were recorded in Brazil. In the 60's resistance to chloroquine was also reported from several areas of the world (Bruce-Chwatt, 1985; Wernsdorfer & Payne, 1991). Resistance of *P. falciparum* to pyrimethamine, was reported shortly after its introduction. However, the combination of pyrimethamine with sulfadoxine (Fansidar) has been used successfully for the treatment of chloroquine resistant malaria for a long time and resistance to this formulation has only been reported from areas where it has been used on a large-scale. Thus, in the 1980s, 90% of all cases failed to respond to this treatment on the Thai-Cambodia border. Resistance to Fansidar has also been reported from other areas. Mefloquine, another recently developed compound used for the treatment of chloroquine resistant malaria, has been routinely used for the treatment of *P.falciparum* malaria in Thailand and the first observation of resistance to mefloquine was from that country (Wernsdorfer & Payne, 1991).
Since the first report of drug resistant *Plasmodium*, it has been the subject of intense study and discussion especially the biological and molecular mechanisms that govern drug resistance, since widespread resistance to antimalarial drugs makes control of malaria increasingly difficult.

1.11.1 Pyrimethamine, Fansidar and Proguanil

Pyrimethamine, a diaminopyrimidine, is very active against growing exo-erythrocytic (EE) stages of *P.falciparum* with limited effect against *P.vivax*. For the other two human malaria parasites it also acts on EE stages but very slowly. There is no evidence that it has any action on the number or morphology of gametocytes, but it inhibits sporogony (Geary *et al*, 1986). It functions by inhibiting the enzyme DHFR thus impeding the metabolism of folic acid. Fansidar which is a combination of pyrimethamine and

sulfadoxine, has the same activity as pyrimethamine alone, but the sulfadoxine is synergic by inhibiting a second enzyme dihydropteroate synthase (DHPS), which is required for the metabolism of p-amino benzoic acid (PABA).

Proguanil belongs to the group of biguanides and has the same role in the chemotherapy of malaria as the diaminopyrimidines since they share a common mechanism of action (Ferone, 1984).

The rapid development of resistance of *Plasmodium falciparum* to proguanil and pyrimethamine is well recognised. Cycloguanil, the active metabolite of proguanil, and pyrimethamine are inhibitors of dihydrofolate reductase (DHFR). Peterson *et al.*, (1990) and Foote *et al.*, (1990) have shown that the resistance of clones of *Plasmodium falciparum* to the antifolates involves alternative mutations in the active site cavity of DHFR. In a further study Peterson *et al.*, (1991) have shown a high incidence of the point mutation (Asn-108) responsible for pyrimethamine resistance among isolates from the Amazon region of Brazil. Most recently Gyang *et al.*, (1992) developed a rapid polymerase chain reaction (PCR) assay for pyrimethamine and cycloguanil resistance based on the invariance of the DHFR point mutations among resistant *P. falciparum* parasites collected over large areas of South America, Africa and Southeast Asia.

1.11.2 Quinine, Mefloquine and Halofantrine

Quinine and mefloquine belong to the group of 4-quinolinemethanols and halofantrine to the group of 9-phenanthrenemethanols (Bruce-Chwatt *et al*, 1981).

These three drugs are effective against the erythrocytic stages of all malaria parasites but ineffective against sporozoites and exoerythrocytic forms (Geary *et al*, 1986; Schuster & Canfiel, 1989).

They have been shown not to cause clumping of malaria pigment, but competitively inhibit chloroquine-induced pigment clumping (CIPC). The studies of Peters, (1987)

suggested that the mode of action of quinine, mefloquine and halofantrine was similar and so explaining the cross resistance seen among the three compounds in rodent malaria and between halofantrine and mefloquine in humans (Gay *et al.*, 1990).

1.11.3 Artemisinin (Qinghaosu)

It is a sesquiterpene lactone which is isolated from the Chinese wormwood plant *Artemisia annua* L. Qinghaosu and its derivates (arthemether and sodium artesunate) are very active against blood schizonts of all species of malaria (blood schizontocide) and also against chloroquine-resistant *P.falciparum* in man (Warhurst, 1987).

It is thought that artemisinin acts primarily on the parasite membrane integrity and so the resistance developed to this drug might involve alterations in the membrane composition (Warhurst, 1987).

1.11.4 Primaquine

This is one of the 8-aminoquinoline drugs and is the least toxic of this group. It is effective against the dormant liver forms (hypnozoitocide), so it is a radical curative drug for *P.vivax* and *P.ovale*. It is also active against gametocytes of all four human *Plasmodium* species. Primaquine also destroys pre-erythrocytic forms of *P.falciparum*, but only in a toxic dose (Mashaal, 1986).

In addition to acting on parasite oxidation-reduction balance, primaquine metabolites effects the oxidative state of the erythrocyte which in turn has affects on the intraerythrocytic malaria parasites (Warhurst, 1987).

Resistance to primaquine has been reported from *in vitro* and clinical studies, which has shown some *P.vivax* strains from South East Asia are less sensitive to primaquine than those from other areas of the world (Warhurst, 1987).

1.11.5 Chloroquine and amodiaquine

Both are 4-aminoquinoline compounds and highly effective

against asexual erythrocytic stages (blood schizontocide), but also destroy gametocytes (gametocytocide) of *P.vivax, P.malariae* and *P.ovale* (Bruce-Chwatt, 1985).

The mode of action of chloroquine is not yet clear but proposed mechanisms include: DNA intercalation, lysosome accumulation and binding to ferriprotoporphorin IX (Meshnick, 1990).

The distribution of *P. falciparum* resistance to chloroquine is worldwide (fig 1.11.5), throughout the malarious areas where *P.falciparum* is found (WHO, 1992).

The nature of resistance of *P. falciparum* to chloroquine is still not clear, but is thought to involve a reduced accumulation of the drug in the food vacuole of the parasite (Krogstad *et al.*, 1987). It has been proposed that this reduced accumulation is due to the efflux of the drug, in a manner similar to that seen in multi-drug resistant cancer cells, and in fact two genes have been identified in *P. falciparum* that are homologous to the genes shown to be responsible for this phenotype in cancer cells (Wilson *et al.*, 1989). One of these *mdr* homologous, *pfmdr*1, has been sequenced (Foote *et al.*, 1989) and the occurrence of a pattern of point mutations was used to predict the correct chloroquine sensitivity of 34 out 36 different isolates of *P. falciparum* (Foote *et al.*, 1990). However, analysis of a genetic cross between a chloroquine-sensitive and a chloroquine-resistant strain suggested that the *pfmdr1* sequence is not linked to chloroquine resistance (Wellems *et al.*, 1990). Further work by Wellems has identified a region on chromosome 7 that segregates in the above mentioned cross, in favour of the chloroquine-resistant isolates (Wellems *et al.*, 1991). More recently, Warhurst and colleagues have shown that resistance may be mediated by differential expression of the two *pfmdr* genes and in field isolates was not related to point mutations in *pfmdr1* (Ekong *et al.*, 1993 and Awad el Kariem *et al.*, 1992). Evidence is also mounting that mefloquine resistance is linked to a high degree of expression of *pfmdr1* (Barnes *et*

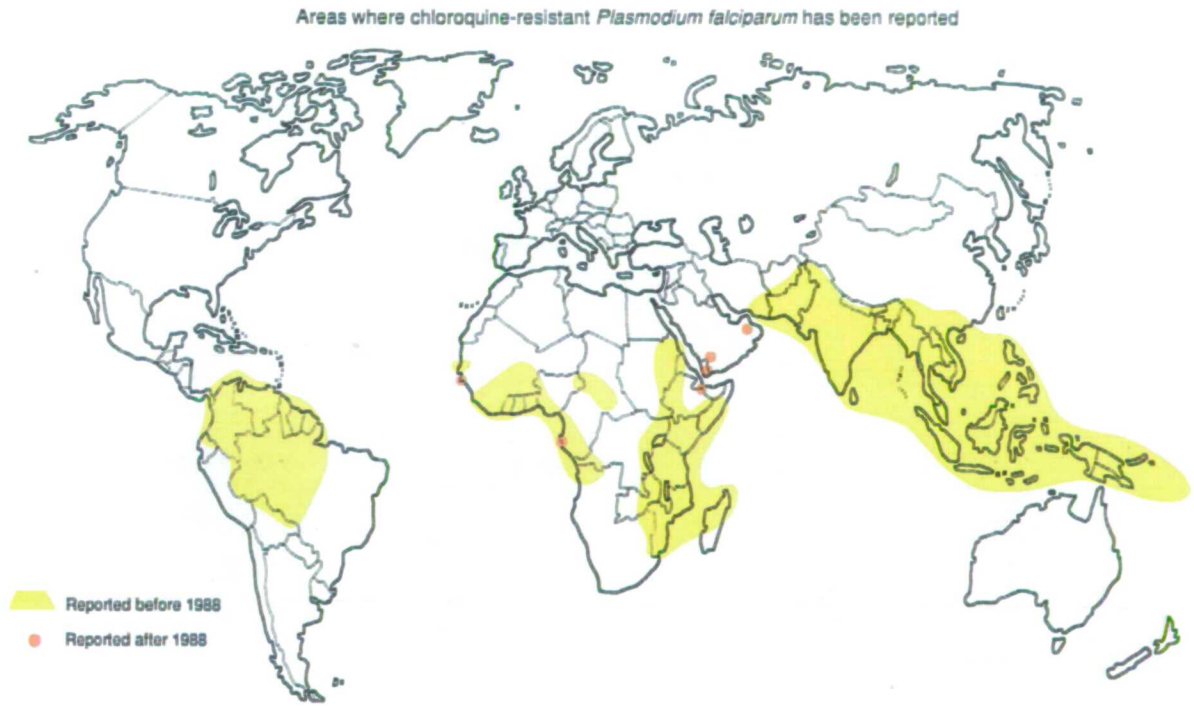


Figure 1.11.5

World distribution of *Plasmodium falciparum* resistance to chloroquine.

al., 1992). Immunological detection methods have shown that the protein product of the *pfmdr1* gene, the P-glycoprotein (Pgh1), is localised on the parasite digestive vacuole during the trophozoite stage of the life-cycle, the stage at which the parasite is normally most susceptible to chloroquine (Cowman *et al.*, 1991). It seems, therefore, that there is a role for the *pfmdr* genes in multi-drug resistance but there is no agreement on exactly what it is.

1.12 Epidemiology

1.12.1 Global malaria

Malaria is very widely distributed in the world and estimates in 1990 (WHO, 1992) show that over 40% of the world population spread among 99 tropical and subtropical countries (Fig. 1.12.1) is at risk of catching malaria.

The global incidence of malaria is estimated to be about 120 million clinical cases per year and about 300 million people are though to carry the parasites.

The four species of human *Plasmodium* are transmitted by female anopheline mosquitoes. Congenital transmission and transmission by blood transfusion or by the sharing of contaminated syringes between drug addicts has also been reported (Lo *et al.*, 1991).

Only a small proportion of the 400 or so species of *Anopheles* are efficient vectors of *Plasmodium*. About 15% of the 400 species feed on man, and with sufficient regularity to maintain endemic infection. There are several different factors that influence the transmission, and temperature, humidity and rainfall play an important role.

Environmental temperature lower than 16° C and higher than 32° C are lethal to the parasite, since at such temperatures no human *Plasmodium* species can develop inside the vector. The optimal temperature for rapid development of the sexual cycle in the vectors is 27° C - this then takes 8 days for *P.vivax* and 11 days for *P.falciparum*. The temperature also affects the vector physiology because the higher the temperature the shorter is the gonotrophic cycle (the

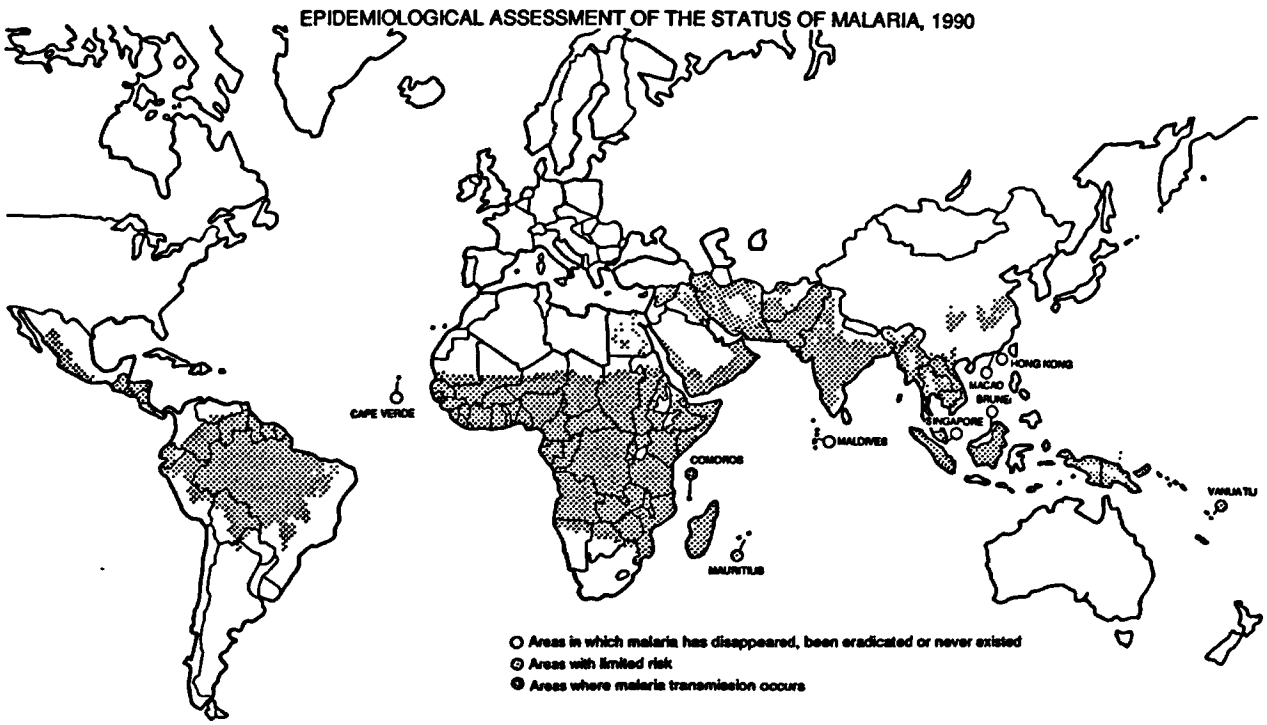


Figure 1.12.1
World distribution of malaria

period of the development of the female *Anopheles* following the blood meal, which begins and ends with egg-laying).
Humidity is also important. A high relative humidity increases the longevity and activity of the vector.
Rainfall also plays a role in malaria transmission due to its direct effect on the temperature and humidity as well in creating favourable breeding sites for the vectors (Mashaal, 1986).
Malaria occurs from as far north as latitude 64°N and to latitude 32°S, at altitudes from 400m below sea level (Dead Sea) to 2800m above sea level (Cochabamba, Bolivia) (Bruce-Chwatt, 1985).
Of the four human *Plasmodium* species, *Plasmodium vivax* is the most widely distributed and causes much debilitating disease; *Plasmodium falciparum* is also widespread and is responsible for the majority of malaria-related deaths because it produces the most severe malaria; *Plasmodium malariae* which also occurs commonly, causes the least severe but most persistent malaria; *Plasmodium ovale* is the least prevalent malaria of the four species and infection is mainly confined to Africa (WHO, 1990). *Plasmodium falciparum* is the predominant species of malaria in tropical Africa, in three countries of Asia west of India, in Bangladesh (middle south Asia), in eastern Asia and Oceania, and in three countries of the Americas.
The malaria mortality in children has fallen in some areas due to the widespread use of antimalarials and to improvements in education and social development. However, in the African region alone WHO registered 800,000 deaths in 1991, but the figure is certainly greater than this because of undereporting of cases. For example, in 1986 the annual malaria mortality in Brazil was estimated at between 6,000 and 10,000 and the total reported period for the Americas was only 1,428 deaths (WHO, 1992).

1.12.1.1 Malaria endemicity

Malaria endemicity can be measured either by the spleen

rate or by the rate of positivity for human *Plasmodium* infections . The enlarged spleen is detected by palpation while the patient is lying down. The parasite rate for human malaria infection is obtained by the reading of individual blood films.

The criteria for endemicity which have been adopted by WHO are measured in the age-group 2-9 years as follows (Bruce-Chwatt, 1985):

Non endemic - no enlarged spleen or positive slides

Hypoendemic - less than 10% present enlarged spleen or positive slides.

Mesoendemic - 11-50% have palpable spleen or positive slides.

Hyperendemic- spleen or parasite rates constantly above 50%. High spleen rates in adults (over 25%).

Holoendemic - high spleen and/or parasite rates (over 75%). Adults with low spleen rates.

The haptoglobin levels has been used as an indicator of malaria transmission. Haptoglobins are alpha-2 globulins, which are synthesised in the liver and whose specific function is to bind free haemoglobin in the plasma.

The release of free haemoglobin into the plasma occurs during haemolytic anaemia and results in rapid saturation of the haptoglobin system and disappearance of free haptoglobin.

Mainly in Africa, low or absent serum haptoglobin has been found frequently and a close correlation between malaria and ahaptoglobinemia has been suggested (Trape & Fribourg-Blanc, 1988 and Sisay *et al.*, 1992). Furthemore, Rougemont *et al.*, (1988); Boreham *et al.*, (1981); Trape *et al.*, (1985) have shown elimination of ahaptoglobinemia by the administration of malaria chemoprophylaxis. As haemolysis may occur in acute malaria an ahaptoglobinemia may be expected during a malaria episode.

1.12.2 Prevalence of malaria in Brazil

According to estimates from WHO (1992) Brazil registered

53% of the 1 057 000 cases recorded from the Americas in 1990. For falciparum malaria infection Brazil was responsible for 75% of all falciparum infections reported from the Americas. *Plasmodium falciparum* was resonsible for 45% of malaria infections in Brazil in 1990 (fig.1.12.2). As shown in fig. 1.12.2a, in 1991 Brazil showed a small decrease in the number of malaria cases recorded in relation to 1990.

1.12.2.1 The distribution and severity of malaria in Brazil

Ninety seven percent of all cases reported in the country in 1990, were from the Amazon region where the highest rate of malaria transmission were found. Three states in this region were responsible for most of the malaria cases: Rondonia (45%), Pará (21%) and Mato Grosso (11%) (PAHO, 1991).

Since 1986 the epidemiological status of malaria in Brazil has been similar to that described above because even in 1986, 96.3% of all cases recorded in the country were concentrated in the Amazon region (PAHO, 1987). In 1986, however, only two states (Pará and Rondonia) accounted for 70% of the all cases in the Amazon region.

Two major social processes are implicated in the increased malarial incidence in the Amazon region: 1- the high and disorderly migratory movement towards mining areas with difficult access, precarious living and working conditions, and the latter include production of stagnant water areas causing increase transmission; 2- the population movements towards areas of subsistence farming where settlements also have very precarious conditions such as inaccessibility, inadequate living conditions and poor health protection (PAHO, 1991). These factors have provided conditions for the continuing transmission and increased risk of contracting malaria.

1.12.2.2 Malaria in Amapá State (AP)

Amapá state in 1990 registered an Annual Parasite Incidence

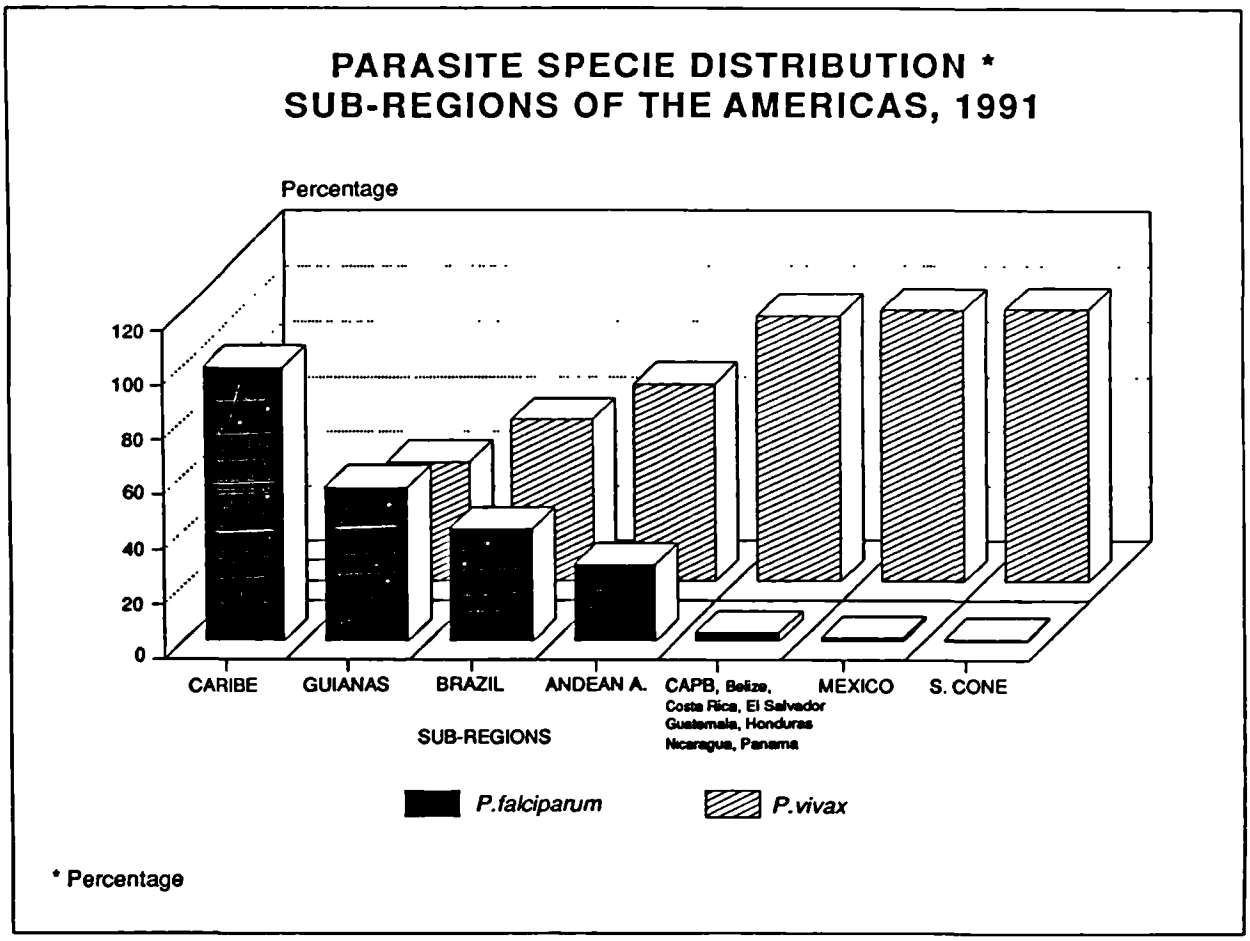


Figure 1.12.2

Comparison between the proportion of malaria caused by *Plasmodium falciparum* or *Plasmodium vivax* in the Americas.

(PAHO, 1992)

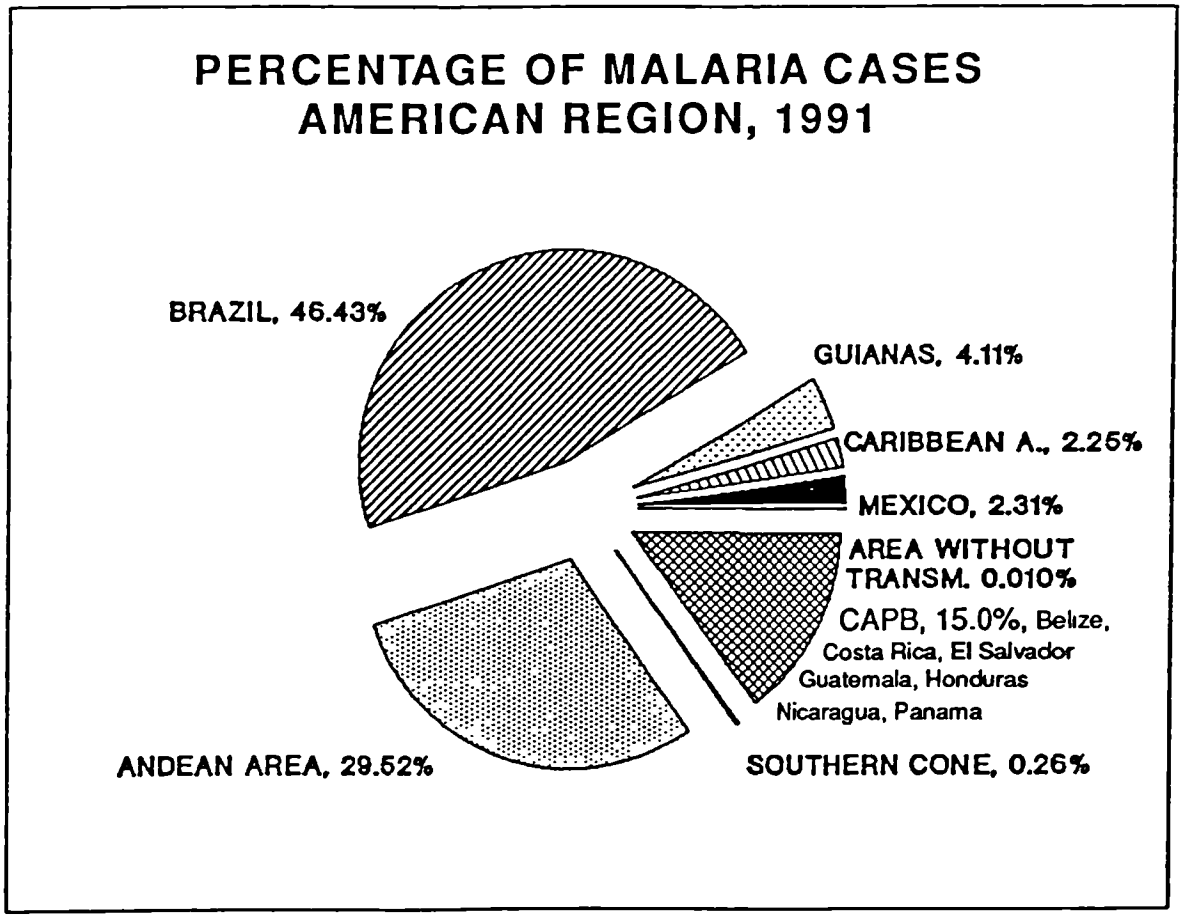


Figure 1.12.2a

Distribution of malaria cases in Americas for 1991
(PAHO, 1992)

(API) of 43.2 per 1,000 (PAHO, 1991). From data kindly provided by the Statistical Sector of the Fundaçâo Nacional de Saúde (FNS) in AP, in 1989 11,156 cases of malaria were registered (positive slides) of which 6,145 were from urban areas and 5,011 from rural areas. For 1991, a total of 9,093 cases were recorded with 4,592 urban and 4,501 rural. For the three first months of 1992 a total of 2,229 cases were recorded with 1,002 urban and 1,227 rural. Throughout these years two municipalities (Macapá and Santana) were the locations where most of the cases were registered, 74.68% in 1989, 72.08% in 1990, 65.13% in 1991, and 70.21% for the first three months of 1992.

1.13 Control of Malaria

Malaria control has been mainly based on the integrated use of measures that target the interruption or reduction of transmission; decreasing and/or stopping man-vector contact, decreasing vector densities and the number of mosquito breeding sites and also eliminating parasites from infected individuals. These measures therefore target towards the people, the vector and the parasite, and they consist of: health education; individual protection (bed nets, mosquito repellents, house screening); application of insecticides and larvicides; use of biological agents such as larvivorous fish (e.g. *Gambusia*) or bacteria pathogenic to the mosquitoes (e.g. *Bacillus thuringiensis*); environmental alterations; chemoprophylaxis and chemotherapeutic treatment of clinical cases.

In 1985 the epidemiological stratification of malaria was recognized as a tool for planning malaria prevention and control activities, although it had emerged as a strategic approach in 1979 (*vide* PAHO, 1992). This new strategy provides the knowledge for the application of the correct measures for malaria prevention and control, through epidemiological studies of individuals and defined social groups, of the risk factors that are responsible for the incidence of malaria at the local level (PAHO, 1991).

The control of malaria should be achievable with the application of those measures, but there are so many complicating factors such as the emergence and spread of resistance to anti-malarial drugs and vector resistance to insecticides, the toxicity of some anti-malarial drugs used for prophylaxis and treatment, the lack of local epidemiological knowledge and/or data, and economic constraints on the application of control measures (high cost, inadequate resource allocation related to the priority endemic areas), that effective control of malaria has not been possible (WHO, 1987). The use of the epidemiological stratification strategy should, however, modify this picture since it is a tool for providing the best understanding of the epidemiological situation, for identifying the factors involved in transmission, and for determination of the most appropriate control measure(s) (PAHO, 1991).

1.14 Conclusion

Malaria continues to be one of the most important parasitic diseases in the world since its morbidity is high and mortality affects mainly children.

Research progress from studies worldwide has given a better understanding of this devastating illness.

Despite the knowledge during recent years, many important problems remain. For example, microscopy is still the method of choice for the diagnosis of malaria; efficient continuous cultivation is only available for *P. falciparum;* the drug resistance continues to be an insoluble difficulty; new drugs have been introduced but their use is under evaluation; control measures have some effect but need improvement to allow decrease in the morbidity of this disease.

1.15 Aims

Based on the fact that Serra do Navio, AP is the only remaining locality in Brazil where chloroquinised salt has

been used for more than 20 years as a preventive measure for malaria infection and this settlement is claimed to be malaria free, this project had the following objective:

1- to obtain a better understanding of the epidemiological status of malaria in the Serra do Navio area;
2- to evaluate the malaria antibody status of the population of Serra do Navio and also of the population of three small localities (control areas) surrounding Serra do Navio;
3- to evaluate if the use of chloroquinised salt is a effective preventive measure.

CHAPTER 2: MATERIAL AND METHODS

2.1 STUDY AREAS

2.1.1 TARGET AREA: Serra do Navio (SNV)

Serra do Navio is a municipality of Macapá city, the Capital of Amapá State. It is 146Km from Macapá (Fig. 2.1.1). The route for reaching Serra do Navio from Macapá is by road (about 3 hours) or by rail (4-5 hours).

Serra do Navio was created in 1947, when the company ICOMI - Indústria e Comércio de Mineraçåo S.A. - received a licence for the exploration of the mineral resource of this area for a period of 50 years. From 1954 to 1957 the company built the industrial project and from 1957-1960 the residential village and the physical basis of the social infra-structure (Gusmåo, 1991). The administrative, industrial, and residential areas are completely isolated from one another. The overall structure was built for a population of about 2 to 2.5 thousand inhabitants. This village has the facilities for covering all population needs, such as: roads, rail, hospital, houses, supermarket, school, communication station, water purification plant, sewage treatment plant, rain water drainage, rubbish incinerator, post-office, bank, pharmacy, police station, cinema, clubs, etc.

The water purification plant comprises the following stages: Aeration, sedimentation, flocculation (using alum), rapid sand filtration, chlorination and fluorination.

The sewage purification plant is a conventional sewage treatment and has the following phases: Pretreatment and primary sedimentation, trickling filters, secondary sedimentation (humus tanks), and effluent discharge.

The residential area is divided up as shown below and all houses are 3 bedroomed houses, very well ventilated to allow for the conditions of climate in the Amazonian region. They have chlorinated and fluorinated water and screened windows and doors, but the size of each type differs, decreasing from type "A" to type "D".

TYPE OF HOUSE	NAME OF THE HOUSES	USERS' GROUP
A	Vila "CC"	Staff members
B	Vila "DD"	Highly specialized workers
C	Vila "A"	Medium-specialized workers
D	Vila "BC"	Non-specialized workers

Serra do Navio was chosen as the target area of the study because of the dietary intake of chloroquinised salt by its population for more than 20 years. The use of chloroquinised salt was introduced in Serra do Navio in 1960 when the Federal Government through the CEM (Programme of Malaria Eradication) started to produce and distribute the salt as a control measure for malaria in all the Amazon region (Gusmão, 1990). The salt was not refined and had in its composition 0.50g% of chloroquine diphosphate and was packed in plastic bags of 30Kg. In April 1961 it was decided to stop the obligation to use this salt in the Amazonian region, but in spite of this the use of the salt continued to finish the stock provided by government. Based on the decrease of the monthly incidence of malaria during the period - 1958 to 1963 - (Table 2.1.1) in October 1963 the company decided to produce and distribute the salt for its two villages, one in Macapá and one in Serra do Navio. The production, preparation, distribution and quality control of the salt and the supervision of its use is carried out by the company itself. The production takes place in a small factory in Macapá and comprises the following stages: trituration, drying, sieving, addition of chloroquine to the refined salt to give a final concentration of 0.40g%, homogenization by gyratory mixer, sampling for testing the chloroquine dosage (Paulini method - Pulini & Pereira, 1963), packing and distribution.
The salt is administered, orally, with the food, since this

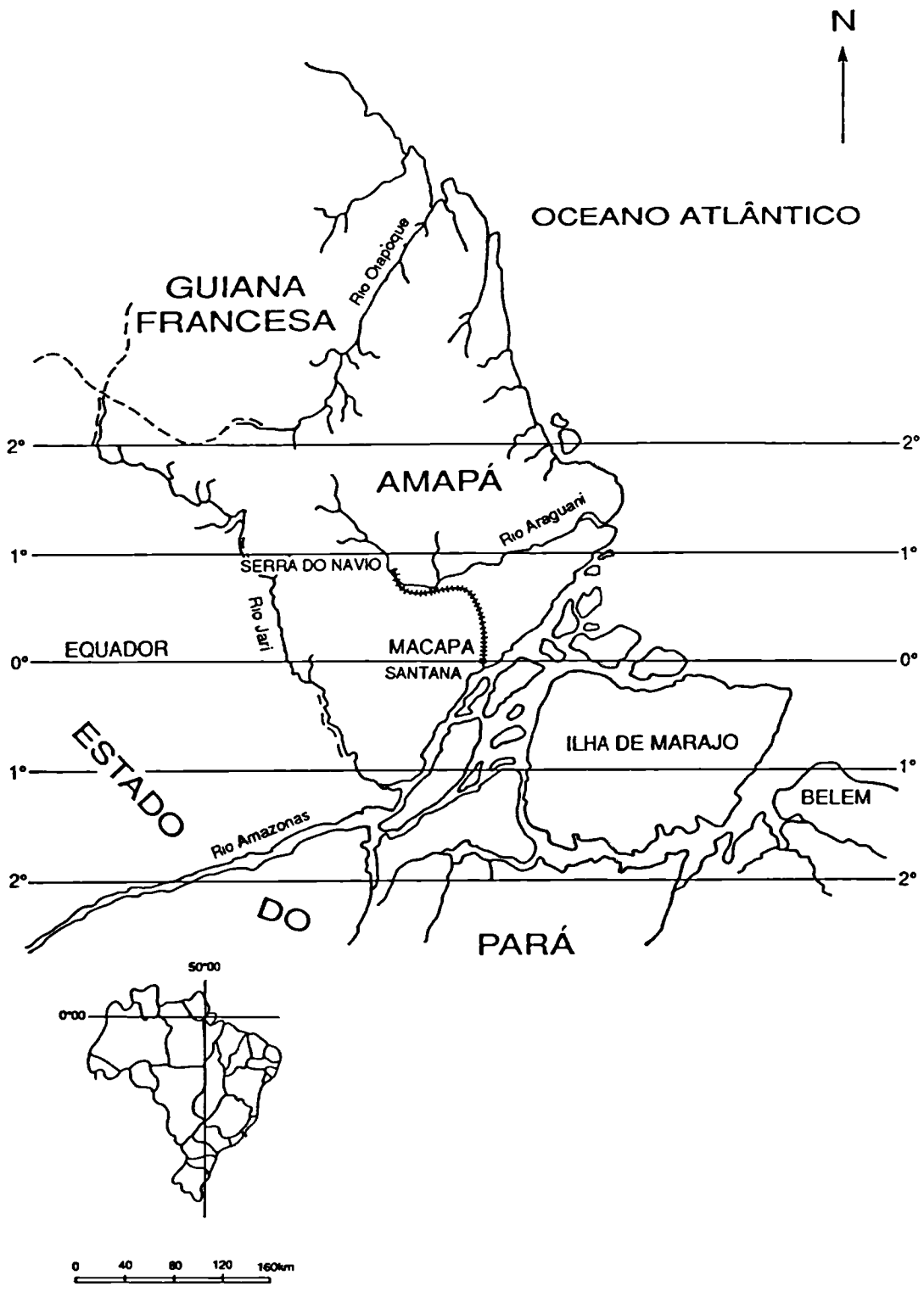


Figure 2.1.1

Map of Amapá State and the railway that links Serra do Navio to Macapá.

TABLE 2.1.1

MALARIA IN THE LOCALITIES OF SERRA DO NAVIO AND PORTO SANTANA-AP

1958 - 1963

YEAR	MONTHS	PROPHYLAXIS	POPULATION #	CASES OF MALARIA	INCIDENCE (%)
1958	1-6 7-12	none pyr*	2.566	252 140	16.4 9.1
1959	1-6 7-12	pyr* pyr*	3.189	84 172	4.4 9.0
1960	1-6 7-12	none chs**	3.476	131 24	4.7 1.7
1961	1-6 7-12	chs** none	3.535	19 418	0.8 23.7
1962	1-6 7-12	cam## none	3.604	316 87	9.7 8.1
1963	1-6 7-12	chs*** chs***	3.819	12 37	0.5 1.6

Population: 1958-1960 - estimate based on the data obtained from the microscopical survey.

1961-1963 - based on the data obtained from the annual demographic census.

*Pyrimethamine: two tablets of 25mg per month

**Chloroquinised salt - without dosage control

Camoprim - 2 tablets of 150mg amodiaquine and 1 tablet of 15mg of primaquine, each two weeks.

***Chloroquinised salt - with dosage control

DATA FROM GUSMÃO, 1990, pp. 135

is the only available salt for cooking. The salt is sold in the only supermarket in Serra do Navio and the control of its use is carried out evèry week in a random sample - 5% of the houses- by collecting samples of salt from the kitchen of each house and testing them for the presence of chloroquine (Paulini method - Paulini & Pereira, 1963). In parallel, urine samples were collected from one or more residents of each house selected (random sample) to confirm the presence of chloroquine by Haskin's method (Haskin, 1958).

2.1.1.1 Geographical characteristics

Latitude - 0 57' 00'' N
Longitude W.Gr. - 52 00' 49''
Altitude - 380 m (highest point)
145 m (houses type "A" and "B")
110 m (houses type "C", "D" and hospital)
Mean annual temperature: 26°C
Mean pluviometric rate: 293.6 mm (Jan-Jun 1990)
129.1 mm (Jul-Dec 1990)

The climate is that of a tropical rainforest usually with a rainy season, during which 85% of the precipitation registered during the year occurs, and a dry season.
The mean temperature does not vary much during the year because of the position of Amapá State on the Equator (Fig. 2.1.1.1), while the daily temperature variation is marked due to the number of hours of sun which is equivalent to the duration of the nights (Amapá State is localized at very low latitudes). The rainfall is lower during the hotter months.
The rainy season is characterised as follows: from December to March - rain every day and several times a day with high intensity but short duration; from April to June - heavy and continuous rain; from July to November - scanty rain.
Serra do Navio itself according to Azevedo, (1967) is classified as "Forest of firm soil" since it is comprised by primary and secondary forest.

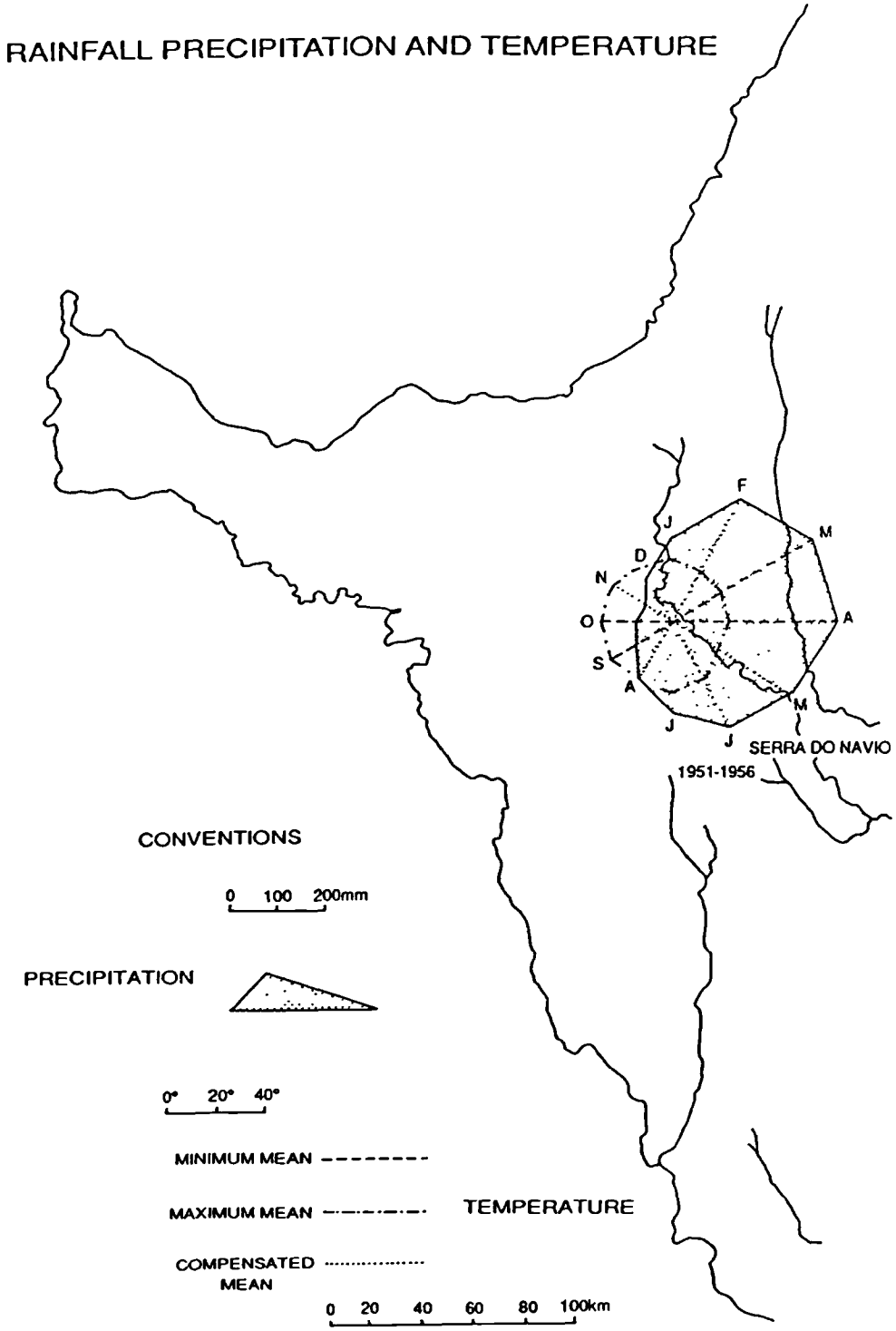


Figure 2.1.1.1

Map of Amapá State with the localization of Serra do Navio and its temperature and rainfall variation.

2.1.1.2 Population characteristics

During the period that this study was carried out (1989-1991), the population of Serra do Navio varied around 2000 inhabitants. They came from anywhere in Brazil and some of them have lived in the area for more than 10 years. The population is comprised of more adults than children and teenagers because the young workers are generally single and the teenagers usually go to other big cities for study.

2.1.1.3 Sample sizes

The size of the sample was established as 15% of the total population of Serra do Navio, that is in 1989, about 250 persons. Random samples were collected by house for each type of "vila". The population group selected was therefore not precisely 15% of the total population because the number of persons per house was variable.

All individuals included in this study had to sign an agreement to allow themselves and their relatives to be studied. For each person, an individual data form was completed and blood and urine samples collected. The urine sample was collected once only while blood samples were taken 3 times during the study, that is once a year (1989-1991).

2.1.2 CONTROL AREAS: Colonia Agua Branca (CAB), Porto Terezinha (PT), Arrependido (ARR)

All three control areas (areas where no chloroquinised salt has been used) are small villages, called localities, and their populations have a standard of living contrasting with that of the population of Serra do Navio (table 2.1.2), in the context of basic needs, such as: treated water, sewage disposal, electricity (except PT),etc. Their houses are made of wood and the roofs are made of zinc panels and sometimes with strong plastic or dry palm tree leaves (Fig. 2.1.2). The Amapari river is their main water source that is used for drinking water, house cleaning, bathing, etc. Rubbish is deposited everywhere, on the

TABLE 2.1.2

DIFFERENCES BETWEEN THE TARGET AND CONTROL AREAS

DIFFERENT POINTS	TARGET AREA (SNV)	CONTROL AREAS (CAB, PT, ARR)
Houses with screened windows and doors	yes	no
School	yes	yes
Supermarket	yes	no
Hospital	yes	no
Water purifiction plant	yes	no
Sewage treatment plant	yes	no
Rain water drainage	yes	no
Rubbish incinerator	yes	no
DDT house spraying programme	yes	yes
Clearance of the small river borders	yes	no
Residential areas, at least, 200 m from the forest	yes	no
Chloroquinised salt programme	yes	no

SNV - Serra do Navio

CAB - Colonia Agua Branca

PT - Porto Terezinha

ARR - Arrependido

Figure 2.1.2

The contrast of house type in the 4 study areas. a. Serra do Navio, b. Colonia Agua Branca and/or Arrependido, c. Porto Terezinha.

river edges, beneath and/or behind the houses. There is no proper disposal of human faeces since they use pit latrines.
CAB and ARR cover a very large area but the house distribution is mainly in patches. The patches in CAB are formed by groups of about 10 houses while those in ARR are of a maximum of 3 houses. PT covers the smallest area but the housing is very concentrated as shown in Fig. 2.1.2.

2.1.2.1 Geographical characteristics

All these localities have the same geographical characteristics as SNV, except for the altitude which is lower, 85m (CAB) and 70m (PT and ARR).

2.1.2.2 Population characteristics

The population of these three localities surrounding Serra do Navio is comprised of people mainly from the areas of the Amazon region and northeast of Brazil. Their main occupation is either free-lance mining (Garimpos) or working for the ICOMI Company.
Population: (data from SUCAM -Ministry of Health, 1990)
CAB - 611 inhabitants
PT - 459 inhabitants
ARR - 80 inhabitants

2.1.2.3 Sample sizes

This was determined as the greatest number of people that it was possible to reach at the time of the sample collections.

2.1.3 Indigenous group in Amapá State.

About 100Km away from the Serra do Navio area there is an indigenous tribe (WAIÅPI). Their houses are made of dry palm trees and they have no sanitary facilities such as treated water and sewage system. They have a very primitive style of living but they are already civilized. The FUNAI (Federal Government organisation for taking care of

indigenous groups) has a group of workers living with them, comprising normally of a medical doctor and/or a nurse, a dentist, a teacher and a manager who speaks their language and is responsible for all subjects concerning their health and well-being.
Their main health problem is malaria and because of this the tribe is included in the DDT house-spraying programme which is carried out by SUCAM (Anti-malaria programme)-Ministry of Health. Malaria diagnosis and treatment are carried out either by FUNAI and/or SUCAM. When the malaria picture is complicated, however, and/or when malaria is epidemic they generally go to the Serra do Navio hospital for diagnosis and treatment.

2.1.3.1 Geographical characteristics
This area has the same characteristics of those described above, except that the altitude is around 60m.

2.1.3.2 Population characteristics
They are native people and are distributed in 16 communities with a total of approximately 900 individuals and a mean of 60 persons per community. Their main occupation is fishing and hunting.

2.1.3.3 Sample size
In this study a small group was included consisting of 12 individuals from the "Aldeia Itaússu" and 15 from the "Aldeia Aramirå" which have populations of 51 and 73 inhabitants, respectively.

2.2 Microscopical examination of blood films
All the individuals included in this study were bled for a 10 ml sample of blood into an EDTA coated tube. Thick and thin films were made from each sample. A drop of blood was placed on the right side of a clean glass microscope slide and a thick film was made by spreading a rectangle (2 cm^2) with the corner of another microscope slide. The thin smear

was produced by touching on the drop with the edge of a microscope slide held at about 45° to the other slide. The blood cells were spread from the middle to the left edge and across the width of the slide with a firm but smooth movement.

The slides were air-dried, thin films were fixed with methanol for 1-2 minutes, and thick films immersed in water for about 10 seconds and immediately counter-stained with methylene blue for 10". They were then placed face down on a staining rack for staining with 10% Giemsa stain (1:9 in buffered dH_2O pH 7.2) for about 25-30 minutes. They were subsequently rinsed gently and air-dried.

Slides were examined with a Zeiss light microscope using oil immersion with a x100 objective. The thick film was observed first for the presence of *Plasmodium* parasites and in this project counts and observations on morphology were derived from thin smears. Parasitaemias were calculated as the number of parasitised erythrocytes per the total number of erythrocytes in 100 fields examined, expressed as a percentage.

2.3 CULTIVATION *IN VITRO*

For the cultivation of *Plasmodium*, we used RPMI 1640 culture medium (CM) as follows:

STEP 1: Incomplete medium:

RPMI 1640	10.40 g/l
HEPES	5.94 g/l
Gentamicin	40.00 µg/ml
Hypoxantine (50 mg/100 ml).....	50.00 ml

Two litres of medium were prepared by dissolving the RPMI 1640 in 1800 ml of distilled water (dH_2O) and adding the other reagents. The pH of the medium was adjusted to 7.2 with 10M NaOH after all the reagents were mixed well by stirring on a magnetic stirrer. The final volume was reached by adding dH_2O up to a volume of 1920ml. The medium was then sterilized on 115ml filter units - 0.22 µm, attached to a vacuum pump in a safety cabinet. Aliquots of

90ml were dispensed in flasks and stored at 37° C for 24h to check their sterility, then stored at -20° C and thawed as needed.

STEP 2:

When needed the number of flasks required were thawed and human serum (A Rh+ or AB Rh+) added to give a final concentration of 10% together with 2.1 ml of a 10% $NaHCO_3$ solution for each 100 ml of CM.

The human serum was obtained either from the Blood Bank (Health Secretary of Pará State-BR) or directly from donors of the Malaria Programme - Evandro Chagas Institute. In both cases their blood was collected in sterile bottles and/or vacutainer tubes (20 ml each). Serum was removed aseptically in the safety cabinet, dispensed in 10 ml aliquots and stored at -20° C until required.

STEP 3:

A suspension of normal human erythrocytes (type O Rh+) was prepared by washing them, twice, with incomplete CM. A 50% suspension of the washed cells was then made in complete CM.

2.3.1 *Plasmodium falciparum*

For culturing and maintenance of *Plasmodium falciparum in vitro* the technique of Trager & Jensen (1976) and Trager (1979) was applied. The initial step was to centrifuge the infected whole blood of the patient for 10 minutes at 1000g and remove the supernatant (SN) and buffy coat. The red blood cells (rbcs) were washed twice by adding an equal volume of incomplete CM and centrifuging with the same conditions described above. The washed cells were then suspended to give a 50% haematocrit (HC). 0.5ml of this suspension was placed in a 60-mm culture dish (Falcon) and 4.5ml of complete CM added to give a culture at 5% HC. To set up a new culture from an existing one it was necessary to calculate the dilution factor which is given by dividing the estimated parasitemia in culture (%) by the required parasitemia (%). This dilution factor was used for diluting

the infected rbcs (50% HC) with the fresh rbcs (50% HC) which were placed in a new culture dish, thus obtaining a final HC of 5%.
The cultures were incubated at 37° C in a candle jar. For this system a desiccator was used equipped with a stopcock and a white candle. The candle was placed in the centre of the desiccator and lit, the cover was put on with the stopcock open, and when the candle went out the stopcock was closed. This system produces a reduced concentration of O_2 and a raised concentration of CO_2. Medium was replaced daily and fresh erythrocytes every third or fourth day.

2.4 Cryopreservation
In this study, glycerolyte 57 solution (appendix -Fenwal Laboratories, Canada) was used as cryopreservation solution.

2.4.1 Cryopreservation of malaria parasites
Wilson *et al.*, (1977) had demonstrated in their study that ring stages of malaria parasites were the stage of choice for the process of direct freezing at -196°C and thawing, and that only cultures rich in rings had to be cryopreserved.
Cultures with 5% or above of ring forms were centrifuged and the SN discarded. Then, twice the cell volume of glycerolyte, drop by drop and under agitation, was added to the cells and each 1.0 ml of the mixture obtained was dispensed into sterile 1.8 ml cryotubes (Nunc) appropriately identified (ID code, date). The cryotubes were then placed in ampoule canes inserted into cardboard sleeves, then canisters, and immediately frozen at -196°C in liquid nitrogen.

2.4.2 Recovery of parasites from liquid nitrogen
The ampoules with the parasites were quickly thawed at 37°C with agitation. The cells, still cold, were transferred into a sterile 10 ml tube and then centrifuged for 2

minutes at 500 g and glycerolyte solution discarded.
Cells were suspended in an equal volume of sterile 3.5% (w/v) NaCl in RPMI 1640 and immediately recentrifuged. Two washes (500 g for 5 min) were carried out in CM, then cells were transferred into a 60 mm culture plate containing 0.5 ml of washed rbcs (see section 2.3) and 4.5 ml of CM. The plates were incubated and maintained as in section 2.3.1.

2.5 Drug resistance test

Pre drug-dosed plates, produced by the WHO Standard Microtest Production Unit, NMCS, Manila, Philippines, were used in this study.
The technique uses 96 well-microplates, in which the wells of the columns identified by alpha codes (B...H) contain increasing anti-malarial drug concentrations. The wells of column A are control wells which have no drug. Those identified by code number (1...12) are for the samples to be tested (Fig. 2.5).
The samples were tested directly from the patient's blood. Starting with a parasitemia of about 0.5-1% rings and early trophozoites. Each blood sample was centrifuged, the SN and buffy coat removed, the cells resuspended in an equal volume of incomplete CM and washed twice at 1000g for 10 minutes. Then the HC was brought up to 5% in complete CM and 50 µl of the sample added to the wells of the same numbered column from the control well to the highest drug concentration. Plates were then rocked gently for about 5 minutes to obtain an homogeneous mixture of the sample with the drugs. The plates were placed in a candle jar, maintaining the humidity by pouring a small amount of water or by placing a piece of wet cotton on the bottom of the jar. They were incubated at 37°C for 24 hours.
For reading the plates were removed from the jar, the medium aspirated from the control well and the remaining red cells mixed and a thin smear made that was stained in Giemsa 20% for 10 minutes. The slide was read as described in section 2.2 and if there were no schizonts, the test

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C	C	C	C	C	C	C	C	C	C
B	1	1	1	1	1	1	1	1	1	1	1	1
C	2	2	2	2	2	2	2	2	2	2	2	2
D	4	4	4	4	4	4	4	4	4	4	4	4
E	8	8	8	8	8	8	8	8	8	8	8	8
F	16	16	16	16	16	16	16	16	16	16	16	16
G	32	32	32	32	32	32	32	32	32	32	32	32
H	64	64	64	64	64	64	64	64	64	64	64	64

Resistance

Figure 2.5

Example of pre-dosed chloroquine plate.

returned back to the jar and incubated for several more hours before repeating the procedure with another control. If schizonts were seen in the control the test was continued as follows: as many microscope slides as the number of tested drugs were made and identified with the date, the sample ID code and the drug name. All the plates were removed from the jar and left resting at an angle of 45° in a safety cabinet until the rbcs had settled on the bottom of the well. The CM was aspirated from each well and a drop (20 µl) of the cells spread on a slide, with the same tip, as a small square. All squares for the same drug were placed on a single slide in two lines of four squares each (Fig. 2.5a). After air-drying for at least 48 hours, the RBCs were stained with Giemsa 2% for 30 minutes.
The slide reading was carried out in the same way as for thick films (see section 2.2) by counting 200 parasites and noting how many were schizonts. Squares that showed no schizonts were considered negative because the drug concentration present in this well inhibited development of the parasites. This concentration is the MIC (minimum inhibitory concentration) and indicated if the sample was sensitive or resistant to the drug(s).

2.5.1 *Plasmodium falciparum*

In this study 4 anti-malarial drugs, amodiaquine (AMQ),quinine (QUI), mefloquine (MQ) and chloroquine (CQ), were tested.
Forty samples were tested but only 18 produced results.
For the limits of resistance and sensitivity see table 2.5.1.

2.6 Indirect immunofluorescence tests (IFATs)

To perform these tests the previously prepared antigen slides (described below) were removed from the -70°C and remained at RT for 30 minutes. Twenty µl of the patient serum sample dilutions prepared in PBS Tween 20 and positive and negative controls, were then applied to the

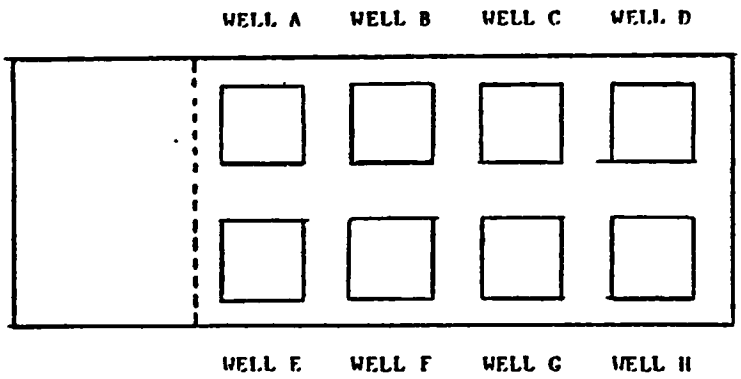


Figure 2.5a
Layout of a microscope slide for reading of antimalarial drug test.

TABLE 2.5.1

CONCENTRATIONS LIMITS (µmol/l of blood) FOR ANTIMALARIAL DRUGS TESTED *IN VITRO*.

(WHO, 1990)

TEST DRUG	SATISFACTORY RESPONSE	INDICATION OF RESISTANCE
	COMPLETE SCHIZONT INHIBITION AT	SCHIZONT GROWTH AT
Amodiaquine	0.4 or less	0.8 or more
Quinine	25.6 or less	51.2 or more
Mefloquine	(*)	12.8 or more
Chloroquine	0.8 or less	1.6 or more

(*) Determination of critical concentration pending (on the basis of comparative *in vivo* and *in vitro* tests).

slides. Slides were incubated at 37°C for 30 minutes in a moist chamber. Two washes with PBS (pH 7.2) were carried out and the slides then covered with the optimum dilutions (1/100) of goat anti-human conjugate (IgG-FITC). Following incubation at 37°C for 30 minutes, two washes in PBS were performed. Finally, they were stained with Evans blue 0.2% in PBS for 5-10 minutes, mounted in 50% glycerol in PBS pH 7.2 and examined under an incident light fluorescent microscope using a X40 objective.
All collected samples were tested by this technique.

2.6.1 *Plasmodium falciparum*

A pool of *Plasmodium falciparum* isolates from the Brazilian Amazon region was grown *in vitro* according to the technique described in section 2.3 (Trager & Jensen, 1976).
Cultures with a 5% of schizontaemia were placed in 50 ml centrifuge tubes and centrifuged at 1000g for about 10 minutes. The SN was removed, the remaining cells washed twice with incomplete CM and resuspended in complete CM bringing up the HC up to 10%. Using a Haemobile, an apparatus that allows the production of eight to twelve drops of the same size and with homogeneous schizont distribution, the slides were prepared. These slides were air-dried, identified with a number for the batch and the date of preparation. Then they were folded in paper tissues, back to back and kept at -70°C.
In this study a screening test was performed with all serum samples diluted at 1:20 in PBS-Tween 20. The positive ones were retested in serial dilutions from 1:40 to 1:640.

2.6.2 *Plasmodium vivax*

The inability to maintain *P.vivax* in long-term culture required use of short-term culture (18-24 hours with 20% human serum) for maturation of the parasite forms. The antigens were therefore produced with a pool of the following infected blood samples directly from the patient: IEC-PV-184/92, IEC-PV-188/92 and IEC-PV-192/92. These

samples originated from Para State, Brazilian Amazon region.
A screening test was performed as for *P.falciparum* and the positive samples were retested in serial dilutions.

2.7 Detection of antibodies against asexual forms of *Plasmodium falciparum* by ELISA.

The antigen for this test was kindly provided by the Laboratório de Soroepidemiologia do Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo -Drs. Antonio Walter Ferreira and Sandra Avila.
The antigen was obtained from cultured *Plasmodium falciparum* (isolates from the Brazilian Amazon), ring forms at about 10% parasitaemia, lysed with saponin and extracted with zwitterionic detergent (ICN Flow).
The 96-well flat bottom microplates (NUNC) were coated with the parasite antigen diluted in carbonate buffer 0.06M pH 9.6 (5 µg/ml as recommended by the providers). The plates were then incubated at 4°C for 18 hours and washed (X3) in PBS 0.1M pH 7.2 and blocked with 5% non-fat powdered milk in carbonate buffer at 37°C for 2 hours. The washes were repeated and the diluted serum (PBS-Tween 20 and 1% non-fat powdered milk, PBS-T-M) were placed in the appropriate wells and the plate incubated at 37°C for 1 hour. The dilution of conjugate at 1:5,000 in PBS-T-M (goat IgG anti-human IgG-peroxidase - Jackson ImmunoResearch laboratories, Inc.) was added and the plate placed at 37°C for another hour. Washes were carried out as before and the chromogenic substrate solution (10mg OPD + 25ml citrate buffer 0.1M pH5.0 + 10 µl H_2O_2 30%) added. The plates were left at RT for 30 minutes protected from the light and the reaction was stopped with 2.5N sulphuric acid. OD was read at 492nm. The Cut Off was determined for each plate by the mean of negative controls plus 2 standard deviations (SD) of this mean.
All samples were prescreened at a dilution of 1:20. The positive sera were retested with serial dilutions from 1:20

to 1:640.

2.8 Detection of antibodies against malaria sporozoite species by ELISA

This ELISA protocol was described by Del Giudice, (1987), and consists of antigen coated and uncoated plates for the sample to be tested.

Coated plates were prepared by dispensing the antigen (1µg/ml in PBS pH 7.2 as recommended by the producer) on each well of a 96-well flat-bottom plates (COSTAR - E.I.A./R.I.A. plate, high binding) and incubating at 4°C overnight. The uncoated plates were prepared by adding PBS pH 7.2 to the plate wells and incubating as for coated plates. The coated and uncoated plates, were blocked with PBS-Tween 20 containing 5% non-fat powdered milk and incubated at RT for 1 hour. They were then washed (X4) with PBS-T and the horseradish peroxidase-conjugated goat IgG anti-human IgG (Jackson ImmunoResearch laboratories, Inc) (1:1,000 in PBS-T-M) was added, and plates incubated at RT for 1 hour. Finally, substrate solution (OPD, 0.4mg/ml in citrate buffer 0.1M pH 5.0 containing 0.01% hydrogen peroxide) was dispensed into the wells. Twenty minutes after the latter step, the reaction was stopped by adding 2.5N H_2SO_4. The OD was determined in a ELISA reader at 492nm.

The final results were obtained from the difference between the OD values of the coated and uncoated plates and the Cut Off was given by the mean of the negative controls plus 3 times the SD of this mean.

The serum samples were assayed in duplicate and first examined at a 1:100 dilution as suggested by the provider of the recombinant protein and/or synthetic peptides.

2.8.1 *Plasmodium falciparum* antigen for sporozoite ELISA

The antigen for *P. falciparum* was the recombinant protein R32LR, was kindly provided by Dr. John Barnwell from University of New York and Dr. Altaf Lal from CDC, Atlanta.

The antigen is the recombinant peptide R32tet32 (consisting of the sequence $(NANP)_{15}$-NVDP$(NANP)_{15}$NVDP, plus the first two amino acids (leucine and arginine - LR) encoded by a tetracycline-resistant gene read out-of-frame (tet32).

2.8.2 *Plasmodium vivax* antigen for sporozoite ELISA

A synthetic peptide $(GDRADGQPA)_3$ that is a nonpeptide derived from the central regions of the prototype *Plasmodium vivax* CS protein (Arnot *et al.*, 1985 and McCutchan *et al.*, 1985) was used in this study as antigen. Dr. Altaf Lal from CDC-Atlanta kindly provided the protein.

2.8.3 *Plasmodium vivax - type 2* antigen for sporozoite ELISA

The 36-mer peptide $(ANGAGNQPG)_3$ was used as antigen for the so-called *Plasmodium vivax* type 2 or variant VK 247.
The repeat unit of VK 247, as in *Plasmodium vivax*, was also a nonapeptide, ANGAGNQPG, but differed from the reactive repeat at six of nine amino acid (AA) positions, as demonstrated by Rosenberg *et al.*, 1989.

2.8.4 *Plasmodium vivax - type 3* antigen for sporozoite ELISA

Qari *et al.*, **(in press)**, have reported the occurrence of a new malaria parasite in "*P. vivax*" infections in humans in Papua New Guinea and Brazil. They also demonstrated that this new "*P. vivax*-like" parasite is closely related to *P. simiovale*.
The antigen used for this variant of *Plasmodium vivax* was the peptide $(APGANQEGGAA)_3$ kindly provided by Dr. Altaf Lal.

2.8.5 *Plasmodium malariae/ Plasmodium brasilianum* antigen for sporozoite ELISA

A 16-mer peptide $(NAAG)_4$ representing the tetramer repeats of *P.malariae/P.brasilianum* (Lal *et al.*,1988) was used as antigen for these species of *Plasmodium* and it was kindly synthesized by the peptide laboratory of the London School

of Hygiene and tropical Medicine.
Lal *et al.*, (1988) had demonstrated a high degree of similarity between the immunodominant region (IDR) of the CS gene of *Plasmodium malariae* and *Plasmodium brasilianum*, and by comparison of the DNA sequence of both genes that the *Plasmodium brasilianum* gene contains 58 and 6 copies of the same major and minor repeats found in the *Plasmodium malariae* gene.

2.9 Determination of haptoglobin levels in children

The haptoglobin assay was carried out with a Radial Immunodiffusion (RID) kit (Serotec-England).
The 14-well plates contain an antiserum in agarose, which had been adsorbed to the wells. The plates were taken out from the fridge and left for 5-10 minutes at RT to allow evaporation of any condensation on the gel. 5 µl of the high, medium and low calibrators (the standards) were applied into separate wells followed by the samples (neat serum) into the remaining wells. After the sample application, the plates were placed in separate lids which were tightly closed and stored at RT for 72 hours, which is the required diffusion time. As the ring diameter is temperature-dependent incubation was at 22°C.
The precipitated ring was measured to the nearest 0.1mm with standard scale provided by the maker.
To calculate the haptoglobin concentration of test samples, a graph was constructed from the results of the standards as follow: on the horizontal axis (abscissa) the antigen concentration of the three standards were plotted and on the vertical axis (ordinate) the squared diameter of their precipitation rings produced by the three calibrators. Then, a line of best fit to the three points was drawn and the tested protein concentrations could be determined from this reference line. This test was performed on one hundred serum samples from children, who were resident in the study areas.

2.10 The Haskin method for detection of chloroquine in urine

This qualitative assay was performed as described by Haskin, (1958). 1.5ml of urine were mixed with 300 µl 10% sodium chloride and 1.5ml chloroform. Then mixture was vortexed and then centrifuged at 2000 rpm for 5 minutes. The chloroform layer was removed and mixed by shaking with 150 µl of methyl orange solution. The presence of chloroquine in the urine sample was detected by the appearance of a definitive yellow colour in the chloroform layer.

METHYL ORANGE SOLUTION consisted of: 100mg methyl orange, 5mg boric acid and 100ml dH_2O, and was prepared by vigorous shaking these reagents and leaving them to stand overnight before filtering and storage at 4°C.

250 urine samples were tested, being 150 from residents in Serra do Navio and 100 from residents in the control areas.

2.11 Quantification of chloroquine by ELISA

The ELISA assay for detection and quantification of chloroquine in human urine and serum was described by Shenton *et al.*, (1988) and Witte *et al.*, (1990). In this study the ELISA developed by Witte *et al.*, (1990) was applied. It is based on the monoclonal antibody (MoAb which recognises the 4-amino-7-chloroquinoline with specificity for the chloroquine molecule (F149-12).

The kits were provided by Koninklijk Instituut Voor de Tropen, Amsterdam, The Netherlands.

The assay consists of coating the microtest plate (COSTAR - E.I.A./R.I.A. plate, high binding) wells with 100 µl of the MoAb F149-12 (1:200 in PBS), and incubating at RT for 2 hours. One quick wash with PBS pH 7.2 with 0.05% Tween-20 followed by two 1 minute washes was performed. 50 µl of serum diluted 1:100 in PBS-T or urine in a serial dilution of 1:10, 1:100 and 1:1,000 were added to each well and mixed with 50 µl of a dilution (1:100 in PBS-T-1% BSA) of a peroxidase-labelled antichloroquine antibody (CQ-7), and

the plate incubated at RT for 1 hour. Finally, the plates were washed (1 quick wash and two 1 minute washes), 100 µl of substrate (0.5mg/ml OPD in phosphate/citrate buffer pH 5.0, 0.3% hydrogen peroxide) were added to each well and the reading was carried out after 30 minutes in a microtitre plate reader at 492nm.
A standard curve (Fig. 2.10) was produced for choosing the best dilution for the conjugate. The procedure consisted of coating the plate with the MoAb as described above. A serial dilution of 11 concentrations of chloroquine (0.156ng/ml to 160 ng/ml) was applied to the rows 2...12, starting from the lowest concentration. Then 50 µl of 4 dilutions (1:200, 1:100, 1:50 and 1:25) of the CQ-7 (conjugate) were added to the rows A...H (each dilution into two rows) starting from the lowest concentration and were then mixed with the chloroquine solutions. The plate was incubated at RT for 1 hour, washed as described above. 100 µl of the substrate were added to each well, the plate incubated at RT, protected from the light, for 30 minutes and read in a plate reader at 492 nm.

2.11.1 Urine

The urine samples were used in the follow dilutions: 1:10, 1:100, 1:1000 in PBS-T.
The urine samples were from the same individuals who had serum samples tested.

2.11.2 Serum

The serum samples were used at 1:100 in PBS-T. All serum samples collected from residents in the study areas were tested.

2.12 Collection of adult and immature forms of mosquitoes of Genus *Anopheles*

This was carried out 4 times, twice in 1990 and twice in 1991, in the wet and dry seasons for about 20 days on each occasion. Different points (sampling places) were chosen

within each study area (SNV, CAB, PT and ARR) and in the forest adjacent to these areas.

2.12.1 Adult forms

All sampling places were visited for at least three days each time that the work was carried out, by a group of 4 people. Catches were made at periodomestic sites (indoors and outdoors) in the residential areas and with light traps (Shannon and CDC) in the forest. Collection periods were 18-21 hours and at least once in each study area a 12 hour collection was made (18-6). Mosquitoes were collected from bare legs, using an aspirator and flashlight (fig. 2.12.1). The group had a rotating catch timetable for preventing a bias of data due to personal differences in attraction to catchers and their skill. In the Serra do Navio area, captures were only made in the adjacent forest. The mosquitoes collected were separated at the time of their collection into maximum groups of ten, per sampling place/time/catch type, in glass tubes.

2.12.2 Immature forms

The collections of immature forms was carried out using dippers (50ml water) in all water collections (fig. 2.12.2): river edges, flooded forest (swamps), pools, creeks, small rivers, ponds, arboreal and epiphytic plants belonging to the Bromeliaceae family, around all study areas. The collection period was: 6-10 am.
All specimens collected were placed into separate tubes containing the breeding site water, split into different stages.

2.13 Identification of adult mosquitoes and larvae

The identification of both forms of anophelines, adult and larvae, was carried out by using the identification keys for adult mosquitoes and anopheline larvae (Faran & Linthicum, 1981).
The adult specimens were examined dead. The specimen can be

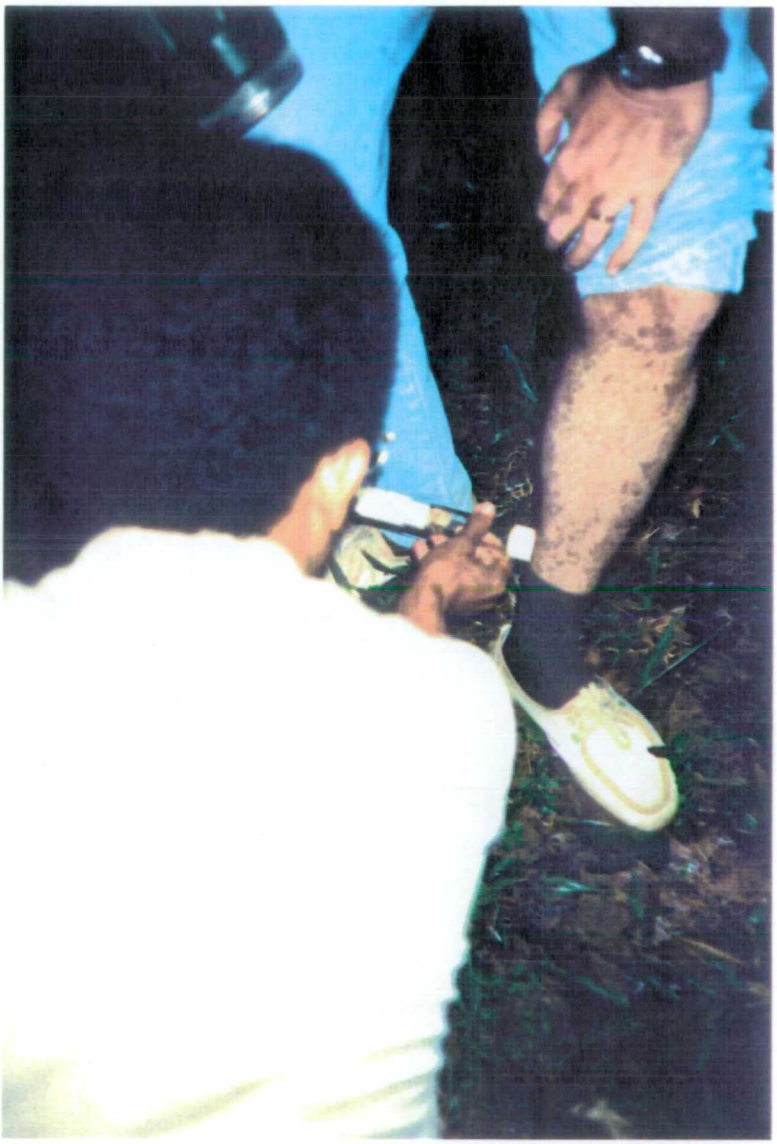


Figure 2.12.1
Collection of mosquitoes on bare legs, using an aspirator and flashlight

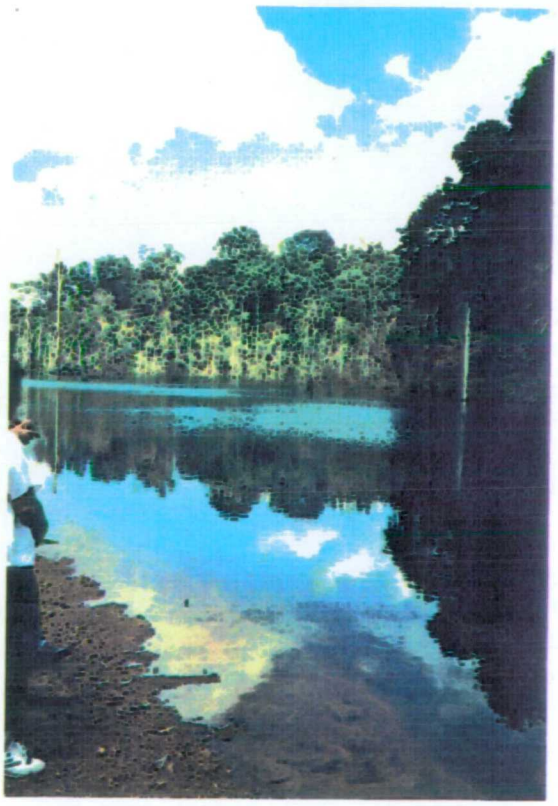


Figure 2.12.2
Examples of collection sites for immature forms of *Anopheles* mosquitoes.

observed either placed on a microscope slide and moved up and down, right and left using entomological forceps and/or needles, or as a pinned specimen that allows it to be moved by rotation, with up and down movements. In both cases the specimen was observed under an entomological microscope.
For reaching the species identification the main characteristics were observed and entered in the identification key.
The anopheline larvae were identified alive or dead, and in this study both types of identification were made. In both cases, the specimen was placed on a microscope slide containing a drop of water to prevent drying. With entomological forceps and/or needles and under a light microscope using X40 objective their main features were observed and entered into the identification key to reach the anopheline species.

2.14 Mounting of adult mosquitoes and larvae

2.14.1 Adult forms

In this study two methods were used for mounting adult mosquitoes: in the first, the specimen was killed in a killer-tube containing vapour of ethyl acetate and then pinned with a special entomological pin (No.0) in the region of the thorax with the head upright. Two labels were prepared on card sheet as rectangles of 1.3 X 0.5 cm, one containing information about the identification such as: name of species, date and who identified it; the other about the catch, where, when and by whom. Both rectangles were also pinned by the same pin of the mosquito specimen and then was placed in an entomological box for storage.
In the second, after the specimen was killed as described above, it was fixed with nail varnish on the narrowest point of a small triangle. It was fixed on the lateral thorax with the head leftward of the triangle. The labels were prepared as already described above and the mosquito labelled and stored in an entomological box.

2.14.2 Immature forms

The specimens were dropped into hot water for killing and then transferred into a series of increasing dilutions of alcohol for dehydration (70% alcohol for about 5 minutes, 90% alcohol for about 5 minutes and finally absolute alcohol for 5 minutes). From the alcohol the specimens were placed into xylene for at least 5 minutes. After that the specimen was transferred to a microscope slide where it was arranged with the head forward and the tail near to the mounter and a coverglass of 20 mm with an appropriate amount of Canada balsam was then put onto the larva. The label on the right side of the slide contained the data for the specimen and that on the left had the name of the specimen. The slide was then placed in an oven for about two weeks at 50°C to be dried.

2.15 Determination of the characteristics of breeding sites

The characteristics of the breeding sites in all study areas were established by observing and/or measuring their size, water pH, water temperature, ambient temperature mean during the collection period, existence of vegetation on the bed, surface and/or edge, type of water, exposure to sunlight, type of stream (weak, strong, absent) and its direction, connection with rivers or other water collections, their distance from the nearest house(s).

2.16 Determination of sporozoite rates by salivary glands detection

The determination of the sporozoite rates was performed on anaesthetized mosquitoes with chloroform or precooled specimens, under a Zeiss entomological microscope by the dissection of their 3 salivary glands. The method applied in this study was the same used by the SUCAM entomological team, which consists of placing the specimen in a drop of physiological solution (saline) on a microscope slide. Using appropriate entomological forceps and/or straight and curved needles, the mosquito head was separated from the

body very carefully in order to allow the salivary glands to come out in the saline without being destroyed or damaged. If this was successful the salivary glands were transferred to another microscope slide containing a drop of a diluted methylene blue solution and a cover glass was placed on the drop and pressed to rupture the glands. In the ruptured glands it was possible to observe the movement of the sporozoites and to count them (heavy or light infection) under a light microscope using oil immersion with X100 objective.

2.17 Determination of sporozoite species in *Anopheles* by ELISA

The kits used to perform this test were obtained through Dr. Robert A. Wirtz, Walter Reed Army Institute of Research and they are produced by Kirkegard & Perry Laboratories.
The method was developed to detect *Plasmodium* species CSP in malaria infected mosquitoes (Wirtz *et al.*, 1987).
The assay is a "sandwich " ELISA and based on the adsorption of the capture MoAb to the wells of "U" shaped well flexible microplate. After the binding of the MoAb to the plate by incubating at RT, the well contents were aspirated and blocking was carried out by adding blocking buffer (appendix 4). Aliquots of ground mosquito thorax (in blocking buffer containing Nonidet-40 -NP-40) were placed in the wells. At the same time positive and negative controls were added to their specific wells. The incubation required 2 hours, and then the contents were aspirated and the wells washed. The appropriate peroxidase-labelled MoAb was added to the wells and the plates were incubated for 1 hour at RT. Aspiration of contents and washes were carried out again and the substrate solution (ABTS + hydrogen peroxidase 3% v/v) was added. The reading of the plates was carried out either visually or at 405-414 nm using an ELISA reader 30 or 60 minutes after the substrate solution had been added.
Preparation of the ground mosquitoes: the head-thorax of

each specimen was separated from the body by cutting it out with a special entomological scissor. The head-thorax was placed in a labelled 1.5ml microtube and ground with a pestle in 50 µl of blocking buffer containing NP-40. The pestle was washed with the same buffer and the rinse collected in the tube to reach a final volume of 250 µl.
The positive mosquitoes for *Plasmodium falciparum* and *Plasmodium vivax* were retested to confirm and estimate the amount of CS protein per mosquito.
Table 2.17 shows the material used in the development of this technique.
As negative controls, male *Anopheles* or *Culex* were used.
The same batches of capture MoAb, peroxidase-labelled MoAb and positive controls were used in all tests.
Not all mosquito specimens were tested for *P. malariae*.
The C.O. was selected as twice the mean of negative control.

2.18 Studies in other localities

2.18.1 The prevalence of mutations associated with pyrimethamine resistance

42 blood samples from individuals in the Amazonian region with falciparum malaria infection were tested for the presence of DHFR-mutations following the protocol described by Peterson *et al.*, (1990) which consists of the extraction of DNA from 300 µl of blood samples cryopreserved directly without *in vitro* cultivation. The thawed samples were then centrifuged and resuspended in 1ml of TSE(100 mM NaCl, 20 mM Tris, 50 mM EDTA, pH 8.0), centrifuged again, and resuspended in 1ml of TSE containing 0.15% saponin. The samples were then incubated for 2-10 minutes at RT and parasites were recovered by centrifugation, resuspended in 500 µl of TSE and lysed by addition of SDS and $NaClO_4$ to give a final concentration of 1% and 0.5 M, respectively. The DNA extraction was performed with phenol:chloroform (2X) and chloroform (1X), and the DNA precipitated with two

TABLE 2.17

MATERIAL USED IN THE DETECTION OF SPOROZOITES IN *ANOPHELES* MOSQUITOES BY ELISA

MATERIAL	*P. falciparum*	*P. vivax*	*P. malariae*	*P.vivax* type 2 (VK247)
MoAb of capture	Pf	Pv210	Pm453	Pv247-499
conjugate	Pf-PER	Pv210 -PER	Pm453-PER	Pv247-PER
positive control	Pf	Pv210	Pm	VK247

volumes of ethanol.
The mutation-specific polymerase chain reaction (PCR) assay was described by Peterson *et al.*, (1991) and was performed by the use of diagnostic primers DIA-3 (3'-specific for Ser-108), DIA-9 (specific for Thr-108) and DIA-12 (specific for Asn-108) conjugated to the counterprimer SP1. For DNA amplification, 45 cycles were used consisting of denaturation for 30 sec(94°), renaturation for 45 sec(56°) and extension for 45 sec(74°). Electrophoresis of the amplified products was carried out in agarose gels containing 1% standard agarose plus 2% NuSieve low melting-point agarose (FMC Bioproducts, Rockland, ME). The PCR products were stained with ethidium bromide and then photographed.
In order to avoid any contamination 3 main measures were adopted: 1- a sample of unparasitized blood was processed in parallel with each sample of infected blood; 2- the processing of blood samples and DNA preparations were carried out in different parts of the laboratory from that used to analyze the PCR reactions; and 3- separate sets of pipettes were used for the analysis of the PCR products.

2.18.2 Paragominas and Jacunda cities, Para State

2.18.2.1 Polymorphism in the circumsporozoite protein of *P. falciparum* and *P. vivax*.

In two trips to Paragominas (1990 and 1991) and one to Jacundá (1991), blood samples infected with malaria parasites were collected.
From each individual epidemiological data and a blood sample were collected. Blood films (thick and thin smears) were made and the morphology of parasites and the parasitaemia were recorded.
The blood samples were then cryopreserved either with glycerolyte (see section 2.4) or without any cryopreservation solution for further DNA studies.
The genomic DNA of the samples was isolated and amplified by PCR, and the PCR products hybridized witth the CS

protein gene probe and sequenced. The amplification was carried with a polymerase chain reaction using specific oligonucleotides as amplifying primers (AL 60 and AL 61 for *P. vivax*, and AL 58 and AL 59 for *P.falciparum* - appendix 5). The amplified fragments were purified, digested with *Eco*RI and *Bam*HI, cloned into the Bluescript plasmid, and transformed into *Escherichia coli*. The hybridization analysis used specific oligonucleotide primers (AL 114 and 116 for *P. vivax*, and AL 52, 53, 3, 9 and 164 for *P.falciparum* - appendix 5). CS gene-specific primers were used to determine the nucleotide sequence of the CS gene by the dideoxynucleotide method (Sanger *et al.*, 1977).

2.18.2.2 Polymorphism in the ookinete vaccine antigen (Pfs25) of *Plasmodium falciparum*

Fourteen blood samplesinfected with *P. falciparum* from individuals from Paragominas were included in this study. The Pfs25 antigen gene of the parasites was amplified from the genomic DNA (Qari *et al.*,1991) using specific oligonucleotide sequences as described above. The amplified DNA fragment (654-bp) was isolated and cloned, and one Pfs25 recombinant per isolate was sequenced.

2.19 Statistical analysis

The statiscal analysis was performed using the computer programme Epi Info - STATCAL Epi calculator - for obtaining the Chi-square and p-values with Yates correction.
For the comparison of the two means the t-test (Student test) was applied.

CHAPTER 3: RESULTS

3.1 Prevalence of infection

The prevalence of malaria infection and the grade of endemicity for all study areas is shown on table 3.1.

3.1.1 Target area: SNV

The study carried out in SNV was longitudinal, where we took 1 sample/year from each individual during 3 years, 1989-1991. The first sample comprised 260 individuals, the second 229 and the third 217, 15.6%, 13.7% and 13.0% of the total population (1667 in 1990), respectively.

The distribution of this population by age and sex is shown in Fig. 3.1.1

In all three samples there were no records of positive slides neither for falciparum malaria infection nor for vivax malaria infection. As shown in table 3.1, the spleen rate of the group aged 2-9 years as well as for adults was zero. Based on these data Serra do Navio was classified as a non-endemic area for malaria infections.

3.1.2 Control areas: CAB; PT; ARR

As shown in table 3.1 the sample sizes for each control area varied. Based on the populations of 1990, the samples were: 41.1% of the population for CAB, 34.8% for PT and 61.2% for ARR.

There was little variation within the populations with respect to standard of living, type of house or basic health service.

The control areas were similar in many respects, except that PT has electricity and a better standard of living since the majority of the workers in this control area work for the ICOMI Company.

The distribution of the population of all control areas by age and sex is shown in Fig. 3.1.1. for comparison between them.

The prevalence was calculated in relation to the population

TABLE 3.1

PREVALENCE OF MALARIA INFECTIONS IN THE STUDY AREAS.

STUDY AREAS	POPULATION (1990)	SAMPLE SIZE	POSITIVITY	PREVALENCE (%)
SNV	1667	1- 260 (15.6%) 2- 226 (13.6%) 3- 215 (12.9%)	0 0 0	- - -
CAB	482	198 (41.1%)	58	29.3
PT	459	160 (34.8%)	10	6.2
ARR	80	49 (61.2%)	10	20.4
IND	124*	27 (21.8%)	11	40.7

SNV - Serra do Navio (target area)

CAB - Colonia Agua Branca

PT - Porto Terezinha

ARR - Arrependido

IND - Indigenous group

* Data from FUNAI

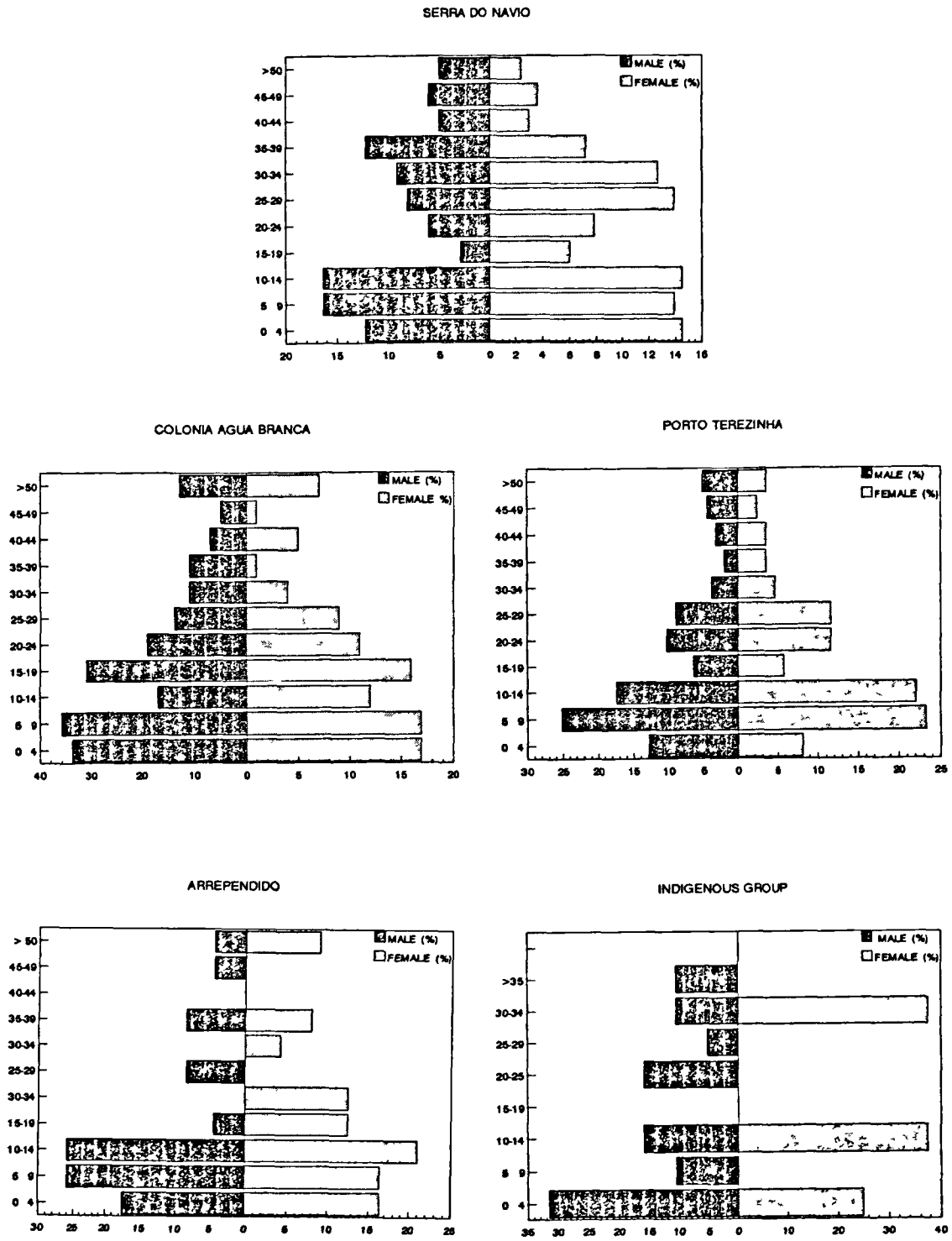


Figure 3.1.1

Distribution of the populations of all study areas by age and sex.

of 1990 by the number of positive slides for malaria parasites at the time of the sample collection. The figures were: CAB - 29.3%; PT - 6.2% and ARR - 20.4% (table 3.1). In CAB and PT more infections were caused by *P. falciparum* than by *P. vivax*, whereas in ARR both had the same prevalence (table 3.1.2).

The spleen rate of the 2-9 year old age group for the three control areas were: CAB - 18.2% (12/66), PT - 6.9% (4/58) and ARR 11.1% (2/18). The parasite rate determined for the same age group was: CAB - 22.7% (15/66), PT - 1.7% (1/58) and ARR 16.7% (3/18).

Both rates, in accordance with WHO classification for endemicity (see section 1.12.1.1), classified the areas as follows: mesoendemic (CAB and ARR) and hypoendemic (PT).

Table 3.1.2 shows the results for malaria parasites (*P. falciparum, P. vivax* and mixed infection) and its relationship with sex, age, spleen rate, malaria history and use of chloroquinised salt and/or individual protection such as bednets and domestic insecticide. The statistical analysis of the figures shown in table 3.1.2 show no correlation between malaria history and either sex or age in the control areas ($p > 0.05$). There was a significant correlation between malaria and spleen rate ($p< 0.05$) for all areas, but this was strongest for ARR ($p= 0.0004$). No correlation was demonstrated between prevalence of malaria and use of chloroquinised salt in the two areas ($p > 0.05$ for both, CAB and PT) where 44.4% and 96.9% people had claimed regular use of this salt. For the correlation between prevalence of malaria and use of individual protection (bednet and/or domestic insecticide), no correlation was found in PT ($p > 0.1$), but in CAB a siginificant correlation was demonstrated ($X^2= 4.79$ and $p= 0.0285557$).

The results shown in table 3.1 give a clear difference in the prevalence of malaria infections in PT as compared to the other control areas.

TABLE 3.1.2

RESULTS OF THE BLOOD FILM FOR MALARIA INFECTION AND ITS CORRELATION WITH SEX, AGE, SPLEEN RATE, MALARIA HISTORY, USE OF CHLOROQUINISED SALT, USE OF INDIVIDUAL PROTECTION.

AREAS	POSITIVITY				SEX		AGE		SR	MH		CHS		INDPRO	
	PF	PV	MX	N	P F/M	N F/M	P C/A	N C/A	P/N	P Y/NO	N Y/NO	P Y/NO	N Y/NO	P Y/NO	N Y/NO
CAB	39	18	1	140	19/39	81/59	18/40	58/82	16/18	54/4	67/73	22/36	67/73	15/43	22/118
PT	7	3	0	150	5/5	82/68	1/9	66/84	7/25	9/1	58/92	8/2	147/3	4/6	48/102
ARR	5	5	0	39	1/9	23/16	4/6	21/18	7/1	8/2	10/29	0/0	0/0	0/0	0/0
IND	10	1	0	16	4/7	4/12	8/3	5/11	9/1	9/2	15/1	0/0	0/0	0/0	0/0

PF - *P. falciparum*; PV - *P. vivax*; MX - *P. falciparum* + *P. vivax*

SR - spleen rate; MH - malaria history; CHS - chloroquinised salt; INDPRO - individual protection

P - positive; N - negative

F - female; M - male

C - children, A - adult

Y - yes; NO - no

CAB - Colonia Agua Branca, PT - Porto Terezinha; ARR - Arrependido, IND - indigenous group.

3.1.3 Indigenous group in Amapá State

The sample which was 21.77% of the total population of the two studied communities (aldeias) had a prevalence of malaria infection of 40.7% (11/27) at the time of collection (table 3.1). *P. falciparum* infection had higher prevalence (90.9%) than *P. vivax* (9.1%) among this sample (table 3.1.2). The distribution of the sample population by age and sex is shown in Fig. 3.1.1.

The spleen rate was 60% among the group aged 2-9 years and 17.6% among the adults. The parasite rate for the same groups was 66.7% and 29.4%, respectively. This area was thus classified as an hyperendemic malaria area.

As for the three control areas a significant correlation was found between malaria history and spleen rate ($p = 0.010$).

3.2 Cultivation *in vitro*

3.2.1 *Plasmodium falciparum*

18 (32.73%) out 55 falciparum infected blood samples were grown with success, since they were maintained for several cycles. The success of culturing samples from the field depends on the level of technical skill as well as the amount of anti-malarial drugs that the samples contain.

3.2.2 *Plasmodium vivax*

All samples with high parasitemia and rich in trophozoite forms (8) were grown for a short-time only to allow the maturation of schizonts.

3.3 Drug tests

Of 17 falciparum malaria infected blood samples tested for their sensitivity to antimalarial drugs, 16 were from the control areas and 1 from the indigenous area. Of the 16, 12 were from CAB (75%), 2 from PT (12.5%) and the other 2 from ARR (12.5%).

The amodiaquine resistance (AMQR) among the samples tested

had a rate of 41.2% (7/17), while chloroquine resistance (CQR) was 82.3% (14/17). There was no sample resistant to either quinine or mefloquine. All AMQ-R samples were also resistant to chloroquine.

No correlation was found between the AMQR and use of chloroquinized salt in both cases tested: all samples (X^2= 0.22 and *p= 0.638*) and CAB samples (X^2= 0.02 and *p= 0.897*). As for AMQ, there was no correlation between CQR and use of chloroquinized salt either in the samples from CAB alone or in all samples tested.

The 2 patients from ARR and the one from IND had not used chloroquinized salt as a preventive measure and all three samples were CQ-R, while the two samples from PT had stated regular use of this salt and their samples were also CQ-R. All CQ-S samples were from CAB. Of the 9 CQ-R samples from CAB, only 3 reported use of chloroquinized salt, while of the 3 CQ-S only one reported its use.

Table 3.3 summarises these data.

We had also carried out some more drug tests with samples from individuals living out of our study areas. They were not included in this study, but we had found chloroquine and amodiaquine resistance and also few quinine resistance among their samples.

3.3.1 *Plasmodium falciparum*

3.3.1.1 Amodiaquine

17 blood samples were tested for amodiaquine sensitivity: 10 were sensitive (58.83%) and 7 resistant (41.17%). These results are shown in table 3.3 where the MIC for each sample is expressed in µmol/l blood.

Among the samples from CAB, 6 were amodiaquine resistant (AMQ- R) and the remaining 6 were amodiaquine sensitive (AMQ-S). Of those from PT (2) were AMQ-S; one of two from ARR was AMQ-S and the other AMQ-R. The only sample from the indigenous group was AMQ-S.

TABLE 3.3

RESULTS OF THE DRUG TESTS OF THE SAMPLES FROM THE STUDY AREAS.

MIC - (μmol/l of blood)

SAMPLE CODE	STUDY AREA	CHS	AMQ	QUI	MQ	CQ
IEC-PF-07/89	CAB	no	0.2 (S)	1.6 (S)	0.4 (S)	3.2 (R)
IEC-PF-08/89	CAB	no	0.2 (S)	3.2 (S)	0.4 (S)	3.2 (R)
IEC-PF-12/89	CAB	no	0.2 (S)	1.6 (S)	0.4 (S)	0.4 (S)
IEC-PF-33/89	CAB	yes	0.8 (R)	6.4 (S)	0.4 (S)	6.4 (R)
IEC-PF-34/89	CAB	yes	0.4 (S)	6.4 (S)	0.4 (S)	6.4 (R)
IEC-PF-38/89	CAB	no	0.4 (S)	3.2 (S)	0.4 (S)	0.8 (S)
IEC-PF-45/90	ARR	no	0.4 (S)	6.4 (S)	0.4 (S)	3.2 (R)
IEC-PF-49/90	PT	yes	0.4 (S)	6.4 (S)	0.4 (S)	3.2 (R)
IEC-PF-53/90	ARR	no	3.2 (R)	6.4 (S)	0.4 (S)	1.6 (R)
IEC-PF-54/90	CAB	no	0.8 (R)	1.6 (S)	0.4 (S)	1.6 (R)
IEC-PF-58/90	CAB	yes	0.8 (R)	1.6 (S)	0.8 (S)	1.6 (R)
IEC-PF-59/90	CAB	no	3.2 (R)	6.4 (S)	0.4 (S)	1.6 (R)
IEC-PF-05/91	CAB	no	1.6 (R)	3.2 (S)	0.4 (S)	1.6 (R)
IEC-PF-06/91	CAB	no	0.2 (S)	3.2 (S)	0.4 (S)	0.8 (S)
IEC-PF-07/91	CAB	no	0.8 (R)	6.4 (S)	0.4 (S)	3.2 (R)
IEC-PF-15/91	IND	no	0.2 (S)	12.8 (S)	3.2 (S)	1.6 (R)
IEC-PF-48/92	PT	yes	0.4 (S)	6.4 (S)	0.4 (S)	3.2 (R)
TOTAL R	-	-	7/17 (41.2%)	0/17	0/17	14/17 (82.3%)

MIC - minimal inhibitory concentration;

CHS - chloroquinised salt

3.3.1.2 Quinine

All 17 samples tested were quinine sensitive (QUI-S). Results are shown in table 3.3.

3.3.1.3 Mefloquine

As for quinine, 100% of the samples were mefloquine sensitive (MQ-S). Table 3.3 shows the results.

3.3.1.4 Chloroquine

Among the 17 samples tested for chloroquine sensitivity 3 (17.6%) of them were chloroquine sensitive (CQ-S) and 14 (82.3%) were chloroquine resistant (CQ-R). 9 out of 12 samples (75%) from CAB were chloroquine resistant (CQ-R) and both samples (100%) from PT and ARR were also CQ-R. The only sample from the indigenous group was also CQ-R (100%).

3.4 Indirect immunofluorescence tests (IFATs)

3.4.1 *Plasmodium falciparum*

The seropositivity for this *Plasmodium* species was higher than for *Plasmodium vivax* in all study areas.

3.4.1.1 Target area (SNV)

In the target area (SNV) there was a seropositivity for this species of *Plasmodium* of 12.3% (32/260) and 19 individuals were positive on one collected sample, 12 on two and 1 on all three.

Table 3.4.1.1 shows the distribution of the titres of positive samples among the population by age group. More than 50% of the positive samples have a positive titre of 1:20, 19.3% of 1:40, 16.1% of 1:80 and 3.2% each of 1:160 and 1:640. There was no positive sample with a titre of 1:320.

71% of the positive samples were from individuals older than 14 years.

There was significant correlation between malaria history and antibody to *P. falciparum* detected by the IFAT (X^2 = 4.20 and $p < 0.05$).

TABLE 3.4.1.1

RESULTS OF THE SEROLOGY OF SNV

a.IFAT - P.falciparum / P.vivax

AGE GROUP	TESTED SAMPLES	(-VE) PF/PV	RECIPROCAL TITRES PF/PV 20	40	80	160	320	640	(+VE) (%)	*GEOMETRIC MEAN TITRE PF/PV
0-1	3	3/3	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-
2-4	32	30/30	2/0	0/0	0/0	0/0	0/0	0/0	6.2/0	4.3/-
5-9	39	35/37	2/2	1/0	0/0	1/0	0/0	0/0	10.2/5.1	4.6/4.3
10-14	41	38/41	2/0	1/0	0/0	0/0	0/0	0/0	7.3/0	4.6/-
> 14	145	123/137	12/2	4/3	5/3	0/0	0/0	1/0	15.2/5.5	5.2/5.4
TOTAL	260	229/250	18/4	6/3	5/3	1/0	0/0	1/0	11.9/3.8	-

b.ELISA - *P.falciparum*

AGE GROUP	TESTED SAMPLES	(-VE)	RECIPROCAL TITRES 20	40	80	160	320	640	(+VE) (%)	*GEOMETRIC MEAN TITRE
0-1	3	2	1	0	0	0	0	0	33.3	4.3
2-4	32	19	11	1	0	1	0	0	40.6	4.6
5-9	39	20	16	3	0	0	0	0	48.7	4.4
10-14	41	28	11	2	0	0	0	0	31.7	4.5
> 14	145	112	27	5	0	0	1	0	22.7	4.6
TOTAL	260	181	66	11	0	1	1	0	30.4	4.5

(-VE) - negative;

(+VE) - positive

PF - *P. falciparum;*

PV - *P. vivax*

* $\log_2$

3.4.1.2 Control areas (CAB, PT and ARR)

For CAB the seropositivity for *P. falciparum* was 46.2% (91/106). The distribution of positive samples by titres is shown in table 3.4.1.2. 73.6% of the positive individuals were adults (>14 years old). The correlation between malaria history and positive IFAT for *P. falciparum* was significant for all groups: children alone, adult alone and all together. As shown in table 3.4.1.2a there was also significant correlation between spleen rate and positivity for IFAT for *P.falciparum* for the group of children alone, for all groups but no correlation was found within the adult group. The correlation between IFAT for *P. falciparum* and positive slides was significant for both groups, children and all ages (table 3.4.1.2a).

Porto Terezinha area had a seropositivity rate for falciparum malaria of 35% (56 positive samples). As for CAB, the positivity is concentrated in the adult group (67.8%).

Distribution of positivity by titres and age group is shown in table 3.4.1.2b.

Table 3.4.1.2c summarises the results of the statistical analysis and shows the correlation between seropositivity and malaria history, spleen rate and positive slide for the group that includes all ages. When analyzed in isolation, neither the children nor the adults show correlation between any of the analyzed variables.

For ARR a seropositivity for *P. falciparum* by IFAT of 26.5% was found. The positivity was higher among the adult group where the rate was 76.9%.

The distribution of the positive samples by titres and age groups is shown in table 3.4.1.2d.

As shown in table 3.4.1.2e only the correlation between seropositivity, and history of malaria within the group of all ages demonstrates significance.

As final analysis, the correlation between the seropositivity with this test and all three variables (MH,

TABLE 3.4.1.2

RESULTS OF THE SEROLOGY OF CAB

a.IFAT- *P.falciparum* X *P.vivax*

AGE GROUP	TESTED SAMPLES	-VE PF/PV	RECIPROCAL TITRES PF/PV 20	40	80	160	320	640	+VE (%)	*GEOMETRIC MEAN TITRE PF/PV
0-1	3	1/2	1/0	0/0	1/1	0/0	0/0	0/0	67/33	5.3/6.3
2-4	30	24/23	0/3	0/0	2/3	1/0	1/1	2/0	20/23	7.8/5.7
5-9	35	26/27	1/5	1/2	2/0	3/0	1/1	1/0	26/22	6.8/5.1
10-14	19	12/16	3/2	0/0	0/1	3/0	1/0	0/0	36/15	6.2/5.0
> 14	110	43/68	16/12	3/7	9/14	10/6	15/2	14/1	61/38	7.0/5.5
TOTAL	197	106/136	21/22	4/9	14/19	17/6	18/4	17/1	46/31	6.9/5.7

b. ELISA - *P.falciparum*

AGE GROUP	TESTED SAMPLES	-VE	RECIPROCAL TITRES 20	40	80	160	320	640	+VE (%)	*GEOMETRIC MEAN TITRE
0-1	3	2	1	0	0	0	0	0	33.3	4.3
2-4	30	13	15	2	0	0	0	0	56.7	4.4
5-9	35	26	6	1	1	1	0	0	25.7	4.9
10-14	19	6	11	0	1	0	0	1	68.4	4.8
> 14	110	51	37	3	8	6	3	2	53.6	5.3
TOTAL	197	98	70	6	10	7	3	3	50.2	5.1

-VE - negative;

+VE - positive

PF - *P. falciparum;*

PV - *P. vivax*

* $\log_2$

TABLE 3.4.1.2a

RESULT OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P. falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

COLONIA AGUA BRANCA (CAB)

CORRELATION	AGE GROUP	CHI-SQUARE	*p VALUE*	SIGNIFICANCE
IFAT X MH	children	14.45	0.0001	yes
	adult	5.48	0.02	yes
	all	21.87	0.0000	yes
IFAT X SR	children	12.46	0.0004	yes
	adult	0.39	0.53	no
	all	7.00	0.008	yes
IFAT X SL	children	13.80	0.0002	yes
	adult	2.74	0.1	no
	all	14.23	0.0002	yes

MH - malaria history

SR - spleen rate

SL - positive slide

children - (0 - 14 years)

adult - (<14 years)

Chi-square and *p values* used with Yates correction.

TABLE 3.4.1.2b

RESULTS OF THE SEROLOGY OF PT

a.IFAT- *P.falciparum / P.vivax*

AGE GROUP	TESTED SAMPLES	(-VE) PF/PV	RECIPROCAL TITRES PF/PV 20	40	80	160	320	640	(+VE) (%)	*GEOMETRIC MEAN TITRE PF/PV
0-1	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2-4	19	14/17	0/0	0/1	2/1	3/0	0/0	0/0	26/10	6.9/5.8
5-9	37	31/37	5/0	1/0	0/0	0/0	0/0	0/0	16/0	4.5/-
10-14	30	23/26	1/2	3/1	1/1	1/0	1/0	0/0	23/13	6.0/5.1
> 14	66	28/48	8/3	5/5	6/6	8/1	4/1	7/2	58/27	6.8/6.2
TOTAL	152	96/128	14/5	9/7	9/8	12/1	5/1	7/2	37/16	6.6/6.0

b.ELISA - *P.falciparum*

AGE GROUP	TESTED	(-VE)	RECIPROCAL TITRES 20	40	80	160	320	640	(+VE) (%)	*GEOMETRIC MEAN TITRE
0-1	0	0	0	0	0	0	0	0	0	-
2-4	18	11	5	0	0	2	0	0	38.9	5.2
5-9	37	22	13	0	1	0	1	0	40.5	4.7
10-14	29	16	12	0	0	0	1	0	44.8	4.6
> 14	64	26	27	2	1	3	0	5	59.3	5.3
TOTAL	148	75	57	2	2	5	2	5	49.2	5.0

(-VE) - negative;

(+VE) - positive

PF - *P. falciparum;*

PV - *P. vivax*

* $\log_2$

TABLE 3.4.1.2c

RESULTS OF THE STASTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P.falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE

PORTO TEREZINHA (PT)

CORRELATION	AGE GROUP	CHI-SQUARE	*p VALUE*	SIGNIFICANCE
IFAT X MH	children	2.07	0.15	no
	adult	0.88	0.35	no
	all	9.32	0.002	yes
IFAT X SR	children	0.01	0.91	no
	adult	0.80	0.37	no
	all	4.88	0.03	yes
IFAT X SL	children	0.59	0.44	no
	adult	0.87	0.35	no
	all	3.98	0.04	yes

MH - malaria history
SR - spleen rate
SL - positive slide
children (0-14 years)
adult (> 14 years)
Chi-square and *p-value* used with Yates correction.

TABLE 3.4.1.2d

RESULTS OF THE SEROLOGY OF ARR

a.IFAT- *P.falciparum / P.vivax*

AGE GROUP	TESTED SAMPLES	(-VE) PF/PV	RECIPROCAL TITRES PF/PV 20	40	80	160	320	640	(+VE) (+)	*GEOMETRIC MEAN TITRE PF/PV
0-1	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	-/-
2-4	9	8/7	0/1	0/0	0/0	1/0	0/1	0/0	11/22	7.3/6.3
5-9	9	8/8	1/1	0/0	0/0	0/0	0/0	0/0	11/11	4.3/4.3
10-14	12	11/10	0/2	0/0	0/0	0/0	0/0	1/0	8/17	9.3/4.3
> 14	19	9/13	6/3	0/1	1/2	0/0	2/0	1/0	47/32	5.8/5.1
TOTAL	49	36/38	7/7	0/1	1/2	1/0	2/1	2/0	26/22	6.1/4.9

b.ELISA - *P.falciparum*

AGE GROUP	TESTED SAMPLES	(-VE)	RECIPROCAL TITRES 20	40	80	160	320	640	(+VE) (%)	*GEOMETRIC MEAN TITRE
0-1	0	0	0	0	0	0	0	0	0	-
2-4	9	9	0	0	0	0	0	0	0	-
5-9	9	9	0	0	0	0	0	0	0	-
10-14	12	12	0	0	0	0	0	0	0	-
> 14	19	16	3	0	0	0	0	0	15.8	4.3
TOTAL	49	46	3	0	0	0	0	0	6.1	4.3

(-VE) - negative; **(+VE)** - positive

PF - *P. falciparum*; **PV** - *P. vivax*

* $\log_2$

TABLE 3.4.1.2e

RESULTS OF THE STASTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P.falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

ARREPENDIDO (ARR)

CORRELATION	AGE GROUP	CHI-SQUARE	*p VALUE*	SIGNIFICANCE
IFA X MH	children	1.64	0.2	no
	adult	0.70	0.4	no
	all	7.64	0.006	yes
IFA X SR	children	2.25	0.1	no
	adult	0.17	0.7	no
	all	2.45	0.1	no
IFA X SL	children	1.64	0.2	no
	adult	0.11	0.7	no
	all	2.34	0.1	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

SR and SL) was performed with the control areas samples taken together due to their similarity. Results are shown in table 3.5.1.4 and the correlations were much more significant mainly for the children and combined (all samples) groups.

3.4.1.3 Indigenous group (IND)

The indigenous group had all samples positive for IFAT of *P.falciparum*. The sample represents 59.2% of people (0-14) and 40.7% of adults.

Thus, the statistical analysis used in this study was not applicable in the case of children for the correlation between seropositivity and MH because all children had positive test and all had reported past malaria infection that is there wasa constant association of the two.

In addition, no correlation was demonstrated, within the age groups, between seropositivity and all variables tested as all samples either with or without history of malaria, enlarged spleen and positive slides had a positive test (IFAT) for *P. falciparum*. Chi-square and *p-values* figures are shown in table 3.4.1.3a.

3.4.2 *Plasmodium vivax*

3.4.2.1 Target area (SNV)

In SNV, the seropositivity for *P. vivax* was 3.8% (10/260). 8/10 (80%) of these positives were from adults.

Among the positive samples eight were positive in one single collection, two for two collections and none for all three collections.

Table 3.4.1 shows the seropositivity of this study area distributed by titres and age groups. The positivity is concentrated in the titres between 1:20 to 1:80.

Since 33 individuals from this area had reported past malaria infection, the statistical analysis between these data and seropositivity was performed. However, no correlation was found.

TABLE 3.4.1.3

RESULTS OF THE SEROLOGY OF IND

a.IFAT- *P.falciparum / P.vivax*

AGE GROUP	TESTED SAMPLES	(-VE) PF/PV	RECIPROCAL TITRES PF/PV						(+VE) (%)	*GEOMETRIC MEAN TITRE PF/PV
			20	40	80	160	320	640		
0-1	0	0/0	0/0	0/0	0/1	0/0	1/0	0/0	100/100	8.3/6.3
2-4	7	0/1	0/0	0/2	1/2	1/1	4/1	1/0	100/86	8.0/6.5
5-9	2	0/0	0/0	0/1	0/0	1/1	1/0	0/0	100/100	6.8/6.3
10-14	6	0/1	0/0	0/1	0/2	2/0	4/2	0/0	100/84	8.0/7.0
> 14	11	0/2	0/1	0/1	0/2	3/2	4/2	4/1	100/82	8.4/7.0
TOTAL	27	0/4	0/1	0/5	1/7	7/4	14/5	5/1	100/85	8.1/6.7

b.ELISA - *P.falciparum*

AGE GROUP	TESTED SAMPLES	(-VE)	RECIPROCAL TITRES						(+VE) (+)	*GEOMETRIC MEAN TITRE
			20	40	80	160	320	640		
0-1	1	0	0	0	0	1	0	0	100	7.3
2-4	7	5	0	0	1	1	0	0	28.6	6.8
5-9	2	2	0	0	0	0	0	0	0	-
10-14	6	4	0	0	1	0	1	0	33.3	7.3
> 14	11	7	1	0	0	1	2	0	36.4	7.0
TOTAL	27	18	1	0	2	3	3	0	33.3	7.1

(-VE) - negative;

(+VE) - positive

PF - *P. falciparum;*

PV - *P. vivax*

* $\log_2$

3.4.2.2 Control areas (CAB, PT and ARR)

The seropositivity (IFAT) for *P. vivax* in CAB was 31%. As for *P. falciparum* the higher positivity was found among the adult group (68.8% - 42/61).

The table 3.4.1.2 also shows the distribution of the seropositivity for this malaria parasite species by titres and age group.

The statistical analysis for the correlation of seropositivity and variables such as malaria history, spleen rate, positive slides were carried out for three age groups, children, adult and all ages. The results are expressed in table 3.4.2.2.

Significant correlation with a history of malaria was found only in the children and combined groups, while with spleen rate no correlation was demonstrated. However, all groups show significant correlation between seropositivity and positive slides either with *P. falciparum* and/or *P. vivax*.

With respect to PT, the seropositivity was 15.8% and 70% of the positive samples were from the adult group. In table 3.4.1.2b all results for serology for *P. vivax* are shown.

Siginificance was demonstrated in both correlations, history of malaria and positive slides vs. seropositivity in two groups: children and all ages. For spleen rate vs seropositivity no correlation was found.

For ARR, where seropositivity was 22.4%, the adults also showed a higher positivity rate (54.5% - 6/11) than children.

Table 3.4.1.2d gives all results of the serology for *P. vivax* by titres within the age groups.

All age groups demonstrated no correlation between their seropositivity for this malaria parasite species and either history of malaria, or spleen rate or positive slides. Results for chi-square and *p-values* are shown in table 3.4.2.2b.

As for *P. falciparum*, the statiscal analysis for IFAT for *P. vivax* was performed for the control areas sample taken

TABLE 3.4.2.2

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P. vivax* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

COLONIA AGUA BRANCA (CAB)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUES*	SIGNIFICANCE
IFAT X MH	children	4.10	0.04	yes
	adult	1.87	0.2	no
	all	8.27	0.004	yes
IFAT X SR	children	0.94	0.3	no
	adult	0.12	0.7	no
	all	1.32	0.2	no
IFAT X SL	children	4.00	0.04	yes
	adult	5.03	0.02	yes
	all	11.35	0.0007	yes

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

TABLE 3.4.2.2a

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P. vivax* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

PORTO TEREZINHA (PT)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUES*	SIGNIFICANCE
IFAT X MH	children	5.45	0.02	yes
	adult	0.07	0.8	no
	all	8.29	0.004	yes
IFAT X SR	children	0.03	0.8	no
	adult	0.12	0.7	no
	all	3.74	0.05	no
IFAT X SL	children	5.32	0.02	yes
	adult	3.18	0.07	no
	all	12.42	0.0004	yes

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

TABLE 3.4.2.2b

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P. vivax* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

ARREPENDIDO (ARR)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUES*	SIGNIFICANCE
IFAT X MH	children	0.21	0.6	no
	adult	1.06	0.3	no
	all	1.68	0.2	no
IFAT X SR	children	0.09	0.8	no
	adult	0.12	0.7	no
	all	0.36	0.5	no
IFAT X SL	children	0.36	0.5	no
	adult	1.71	0.2	no
	all	3.75	0.05	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

together and as shown in table 3.5.1.4, and the significance of the positive correlations were stronger that when the areas were analysed separately. Nevertheless, the adult group continues showing no correlation for seroporitivity vs. malaria history, but had demonstrate correlation of seropositivity with spleen rate, which had not been found for any of the areas separately.

3.4.2.3 Indigenous group (IND)

This group showed a lower seropositivity for *P. vivax* (85.2%) than for *P. falciparum* (100%).

In contrast with all other study areas and in agreement with the results for *P. falciparum*, this group had a higher positivity among children (60.9% - 14/23).

As shown in table 3.4.1.3, where distribution by titres and age groups is given, the titres are distributed among those higher than 1:20.

Table 3.4.2.3 shows the results obtained from statistical analysis for the same variables tested in the other areas. For all variables and all analyzed age groups no correlation was demonstrated.

For the children, the correlation between seropositivity and history of malaria was significant since all children had past malaria infection and 87.5% (14/16) of them had positive test for *P. vivax* (X^2= 4.90 and *p-value= 0.027*).

3.4.3 Infection rate

Draper *et al.*, (1972) produced a mathematical model in which it is possible to predict the probability of an individual being infected during on year within a certain area (appendix 4). The model is based on the fact that once an individual is infected he remains serologically positive, thus the pobability of an infected individual being infected is constant in a small interval of time. He drew standard lines of the probability being infected each year (R) in a $\log_2$ 2 cycles graph paper which, from the

TABLE 3.4.2.3

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (IFAT) FOR *P. vivax* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

INDIGENOUS GROUP (IND)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUES*	SIGNIFICANCE
IFAT X HM	children	4.90	0.03	yes
	adult	0.36	0.5	no
	all	0.34	0.6	no
IFAT X SR	children	0.11	0.8	no
	adult	0.13	0.7	no
	all	0.02	0.9	no
IFAT X SL	children	0.00	1.0	no
	adult	0.11	0.7	no
	all	0.03	0.9	no

ND - not done.
MH - malaria history
SR - spleen rate
SL - positive slide
children (0-14 years)
adult (> 14years)
Chi-square and *p-values* used with Yates correction

proportion of the population with antibodies at different ages, can be used to predict approximately the transmission in a population of an area.
The results of IFAT tests showed the probability of an individual being infected in all study areas (fig. 3.4.3). The transmission in SNV (R < 1%) is lower than the control areas (R = 1 - 5%), but all areas have low transmission levels.

3.5 ELISA with serum samples

3.5.1 Detection of antibodies against asexual forms of *Plasmodium falciparum*

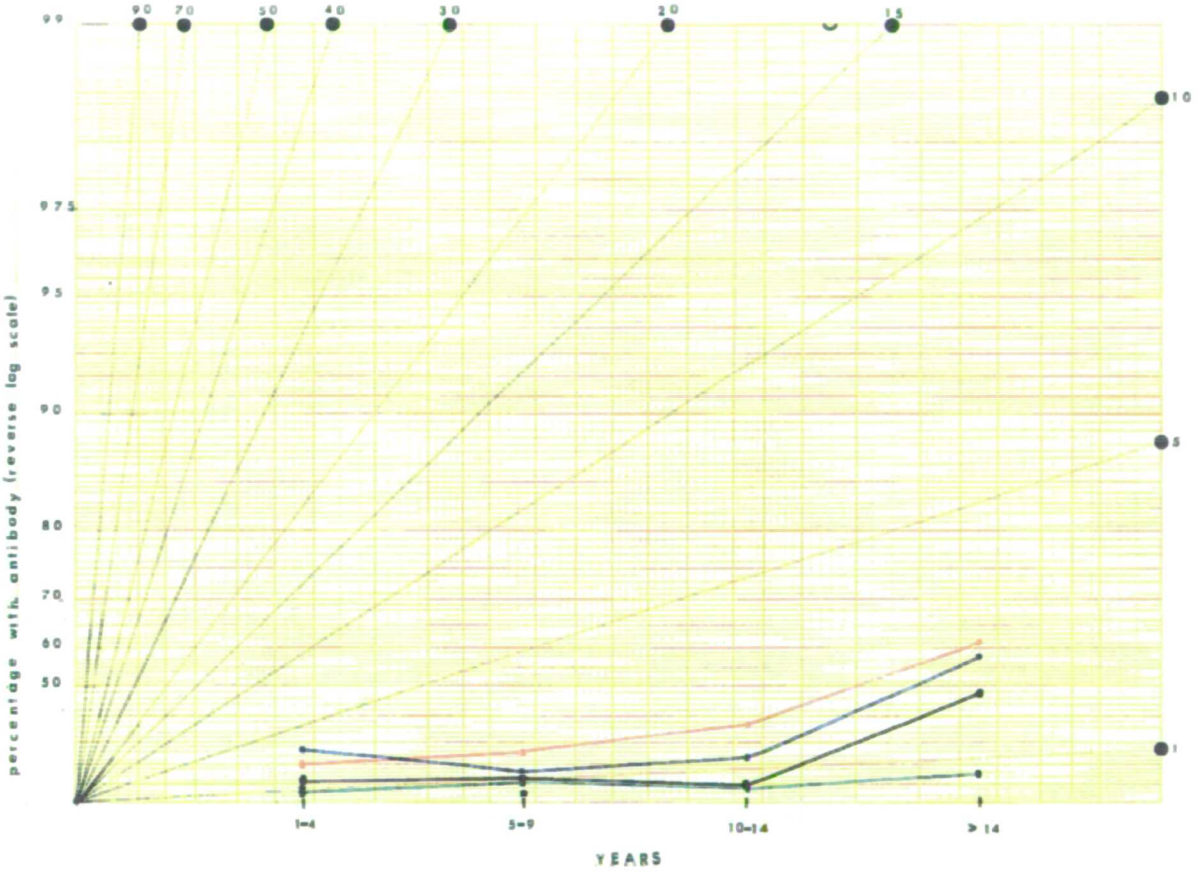
The study of antibodies against asexual forms of *Plasmodium falciparum* gave a rate of 43.35% (114/263) of positivity.

3.5.1.1 Target area (SNV)

The rate of positivity for *P. falciparum* by ELISA for this study area was 30.4% (79/260). Of those, 44 were positive in one single collection, 30 for two and 5 for all three.
Contrasting with the IFAT results for *P. falciparum*, this test had a higher seropositivity among the children (57% - 45/79).
The distribution of titres shown in table 3.4.1 demonstrates that most titres were low; 83.5% were positive for at a titre 1:20.
There was no correlation between seropositivity and malaria history for any age group since the *p-values* were higher than 0.05 (*p= 0.92, 0.15* and *0.50* for children, adults and all age groups respectively).

3.5.1.2 Control areas (CAB, PT and ARR)

The *P. falciparum* serology by ELISA in CAB had a positivity of 50.2% (99/197) and as for SNV the group with the lowest titre (1:20) was the largest.
As in the IFA test the adult group had higher positivity than children (59.6% - 59/99).
Applying statistical methods it was demonstrated that there



● SNV
● CAB
● PT
● ARR

Figure 3.4.3

Draper's method for the probability of being infected by malaria parasites for each study area based on the level of antibodies detected by IFAT.

was no correlation between the ELISA seropositivity and any of the variables tested in any of the age groups (table 3.5.1.2).
49.2% was the positive rate for *P.falciparum* in PT. Low titres around 1:20 were most common (table 3.4.1.2b) and there were more positives in the adult group (52% - 38/73). In this study area no correlation between seropositivity and the variables tested, malaria history, spleen rate and positive slide was demontrated for all three age groups (table 3.5.1.2a).
ARR was the study area where the positivity was the lowest (6.1% - 3/49). The three positive samples were from adults and had titres of 1:20. These results are shown in table 3.4.1.2d.
The statistical methods were not applicable to the analysis among the children group because their tests were negative whether or not they had had past malaria infection, enlarged spleen or positive slides. As shown in table 3.5.1.2b, for the other age groups no correlation was demonstrated between the seropositivity and all variables tested.
However, in order to carry out the statistical analysis the samples from all control areas were taken together, but eventhough no correlation was found between positivity of this test and all variables tested (table 3.5.1.4).

3.5.1.3 Indigenous group (IND)
33.3% of the samples were positive, and as for IFAT children had higher positivity (55.5% - 5/9). In contrast to the other areas the positive titres were above 1:80 (table 3.4.1.3).
The table 3.5.1.3 summarises the results of the statistical analysis and demonstrates no correlation between seropositivity and the variables, malaria history, spleen rate and positive slide.
Among the children, for the same reason as for ARR, the correlation between seropositivity and malaria history was

TABLE 3.5.1.2

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (ELISA) FOR *P. falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

COLONIA AGUA BRANCA (CAB)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUE*	SIGNIFICANCE
EB X HM	children	0.76	0.4	no
	adult	0.03	0.9	no
	all	0.62	0.4	no
EB X SR	children	0.39	0.5	no
	adult	0.52	0.5	no
	all	0.00	1.0	no
EB X SL	children	0.39	0.5	no
	adult	0.08	0.8	no
	all	0.67	0.4	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates corrected

TABLE 3.5.1.2a

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (ELISA) FOR *P. falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

PORTO TEREZINHA (PT)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUE*	SIGNIFICANCE
EB X HM	children	0.04	0.8	no
	adult	0.33	0.6	no
	all	0.85	0.3	no
EB X SR	children	0.06	0.8	no
	adult	0.02	0.9	no
	all	0.12	0.8	no
EB X SL	children	0.24	0.6	no
	adult	0.11	0.7	no
	all	0.06	0.8	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates corrected

TABLE 3.5.1.2b

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (ELISA) FOR *P. falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

ARREPENDIDO (ARR)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUE*	SIGNIFICANCE
IFAT X MH	children	nd	nd	-
	adult	0.20	0.6	no
	all	2.71	0.1	no
IFAT X SR	children	nd	nd	-
	adult	0.01	0.9	no
	all	0.00	0.9	no
IFAT X SL	children	nd	nd	-
	adult	0.53	0.4	no
	all	0.79	0.4	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

TABLE 3.5.1.3

RESULTS OF THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN SEROLOGY (ELISA) FOR *P. falciparum* AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE.

INDIGENOUS GROUP (IND)

CORRELATION	AGE GROUP	CHI-SQUARE	*p-VALUE*	SIGNIFICANCE
EB X MH	children	nd	nd	no
	adult	0.00	0.9	no
	all	0.00	1.0	no
EB X SR	children	0.19	0.7	no
	adult	0.00	0.9	no
	all	0.02	0.9	no
EB X SL	children	0.18	0.7	no
	adult	0.00	0.9	no
	all	0.04	0.8	no

MH - malaria history

SR - spleen rate

SL - positive slide

children (0-14 years)

adult (> 14years)

Chi-square and *p-values* used with Yates correction

not tested.

3.5.1.4 Correlation among the immunological tests

Finally, statistical analysis was carried for all three immunological test for detection of antibodies against blood forms (IFAT for either *P. falciparum* and *P. vivax*, or ELISA) of the control areas samples taken togheter. The results are shown in table 3.5.1.4, and demonstrate a different result from those obtained from the analysis of each area separately.

3.5.2 Detection of antibodies against malaria sporozoite repetitive CS antigens

Table 3.5.2 shows the results of this ELISA test for each study area and sample. Also is shown the positivity by *Plasmodium* species and the number of different positive species by sample.

SNV had a positive rate of 39.6% and as for the other immunological tests the adults showed higher titres (24.6% - 64/260) than children (15% - 39/260).

CAB was the study area where the highest positive rate was obtained (60% - 117/195). Age was not correlated with the seropositivity since the prevalence of anti-sporozoite antibodies was similar in both groups (30.2% for adults and 29.2% for children).

As for CAB, PT had similar seropositivity for both age groups (18.7% for adults and 16.8% for children), while IND had equal rate for the two age groups (18.5%) and ARR had a higher rate for children (40.8%) than adults (10.2%).

In table 3.5.2 the positive rate for all areas is shown and the range that was from 23.1 to 37.0%, excluding CAB.

The statistical analysis for correlating reported intake of the chloroquinised salt with protection against malaria infection was performed as follow: within the group of individuals who had positive test for any of the tested CS antigen it was taken two subgroups, one that had reported the intake of the salt and one that not. From both

TABLE 3.5.1.4

THE STATISTICAL ANALYSIS OF THE CORRELATION BETWEEN THE POSITIVITY OF THE IMMUNOLOGICAL TESTS (IFAT AND ELISA FOR BLOOD FORMS) AND MALARIA HISTORY, SPLEEN RATE AND POSITIVE SLIDE OF THE CONTROL AREAS SAMPLES TAKEN TOGETHER.

CORRELATION	CHI-SQUARE			*p-VALUE*			SIGNIFICANCE		
	CH	AD	ALL	CH	AD	ALL	CH	AD	ALL
IF X MH	16.8	4.0	30.2	0.00004	0.03	0.00	YES	YES	YES
IF X SR	13.1	4.1	30.9	0.0003	0.04	0.00	YES	YES	YES
IF X SL	27.1	3.1	29.1	0.00	0.07	0.00	YES	NO	YES
IV X MH	7.9	1.0	20.9	0.005	0.3	0.001	YES	NO	YES
IV X SR	18.8	5.4	22.6	0.00	0.02	0.00	YES	YES	YES
IV X SL	6.4	4.7	4.9	0.01	0.03	0.00	YES	YES	YES
EB X MH	0.3	0.1	1.9	0.6	0.7	0.2	NO	NO	NO
EB X SR	0.7	0.0	0.4	0.4	0.9	0.5	NO	NO	NO
EB X SL	0.6	0.2	1.8	0.4	0.7	0.2	NO	NO	NO

IF - IFAT for *P. falciparum*

IV - IFAT for *P. vivax*

EB - ELISA for blood forms of *P. falciparum*

MH - malaria history; SR - spleen rate; SL - positive slide

CH - children

AD - adult

Chi-square and *p-values* used with Yates correction

subgroups it was taken the number of individuals who had or not positive slides either for *P. falciparum* or/and for *P. vivax*, see below. Thus, it was demonstrate non-significant correlation for CAB alone, but when taken together with PT, there was significant correlation between the intake of chloroquinised salt and protection (X^2= 10.77 and *p= 0.001*).

CHS	**CS pos. test**	**result of slide**		**correlation**
		pos	neg	
yes (243)	94	13	81	13/243
not (115)	74	21	53	21/115

As shown in table 3.5.2 antibodies against the CS of *P. falciparum* and *P. malariae* were the most and the least prevalent among the total sample, respectively.

We tested our samples for other anti *P. vivax*, anti *P. vivax* type 2 and anti *P. vivax*-like (type 3) CS. The distribution of the samples that were positive for all three types together or for eachof the two is shown in table 3.5.2a. All study areas had positive samples for all three types and only ARR and IND areas had no positive samples for type 1 and type 2 together.

It was observed that within SNV some whole families had positive tests for the same species of malaria parasite sporozoites which suggests that they had been exposed to the same source of parasites.

The number of positive samples for the detection of antibodies against the malaria parasites CS antigen and those that were also positive for either IFAT for both *P. falciparum* and *P. vivax* or for ELISA of the blood forms is shown in table 3.5.2b.

In addition, statistical analysis of these correlation was performed all samples taken together and no correlation was demonstrated for the positivity of each two tests within each type of CS antigen, except for the correlation between the CS antigen of *P. vivax* type 1 and IFAT *P. vivax* and ELISA for *P. falciparum* blood forms where significant correlation was demonstrated (X^2= 45.56 and *p= 0.00*; X^2=

TABLE 3.5.2

RESULTS OF THE ELISA TEST FOR THE DETECTION OF ANTI-MALARIA SPOROZOITES ANTIBODIES BY STUDY AREA, BY SPECIES AND BY MULTIPLE ANTIBODIES.

AREAS	TESTED SAMPLE	(+VE) (%)	RESULTS					No. OFINDIVIDUALS POSITIVE FOR 1-5 CATEGORIES				
			PF	PV_1	PV_2	PV_3	PM	1	2	3	4	5
SNV_1	260	73 (28.1)	28	23	16	26	15	53	11	5	3	1
SNV_2	229	53 (23.1)	21	23	16	23	14	24	19	6	3	1
SNV_3	217	53 (24.4)	19	14	15	20	10	33	16	3	0	1
CAB	195	117 (60.0)	69	54	47	48	5	51	33	26	7	0
PT	155	56 (36.1)	19	19	29	23	3	34	12	5	4	1
ARR	49	17 (34.7)	11	4	5	10	7	8	4	0	4	1
IND	27	10 (37.0)	5	2	6	4	2	5	2	2	1	0
TOTAL	1132	379 (33.4)	172	139	134	154	56	208	97	47	22	5

(+VE) - positive

PF - *P. falciparum*, PV_1 - *P. vivax* - type 1; PV_2 - *P. vivax* - type 2;

PV_3 - *P. vivax* - type 3; PM - *P. malariae/P.brasilianum*

$SNV_{1,2,3}$ - Serra do Navio samples 1, 2 and 3

CAB - Colonia Agua Branca; PT - Porto Terezinha

ARR - Arrependido; IND - Indigenous area

TABLE 3.5.2a

DISTRIBUTION OF POSITIVE SAMPLES FOR 2 OR ALL TYPES OF *P. vivax* BY AREA

STUDY AREAS	ALL 3 TYPES	TYPE 1 + 2	TYPE 1 + 3	TYPE 2 + 3
SNV_1	3	4	4	3
SNV_2	5	4	4	4
SNV_3	1	2	4	2
CAB	15	7	10	14
PT	10	4	1	3
ARR	3	0	1	2
IND	1	0	1	1

$SNV_{1,2,3}$ - Serra do Navio samples 1, 2 and 3

CAB - Colonia Agua Branca

PT - Porto Terezinha

ARR - Arrependido

IND - Indigenous area

TABLE 3.5.2b.

THE RELATIONSHIP OF THE RESULTS OBTAINED BY ELISA FOR MALARIA PARASITES SPOROZOITES AND THOSE BY IFAT AND ELISA FOR BLOOD FORMS IN ALL STUDY AREAS.

SPECIES	RELATION	STUDY AREAS					TOTAL
		SNV	CAB	PT	ARR	IND	
CSF	IF	7	30	8	4	5	54
	IV	1	22	4	1	4	32
	EB	22	18	12	1	1	54
CSV	IF	8	28	11	2	2	51
	IV	3	19	3	1	0	26
	EB	28	33	9	1	1	72
CSK	IF	8	19	16	1	6	46
	IV	2	17	5	0	5	29
	EB	14	19	11	0	1	45
CSN	IF	8	18	14	2	4	46
	IV	4	15	4	1	2	26
	EB	22	23	15	1	2	63
SCM	IF	6	5	0	2	2	15
	IV	2	2	0	1	1	5
	EB	5	3	2	1	1	12

SNV - Serra do Navio; CAB - Colonia Agua Branca

PT - Porto Terezinha; ARR - Arrependido; IND - Indigenous group

CSF, CSV, CSK, CSN and CSM - CS antigen for *P. falciparum*, *P. vivax*-type 1, *P. vivax*-type 2, *P. vivax*-type 3 and *P. malariae*, respectively.

IF - IFAT for *P. falciparum*

IV - IFAT for *P. vivax*

EB - ELISA for *P. falciaprum* blood forms

9.16 and *p= 0.002*, respectively). This results shown a very strong correlation for the first relationship vs IFAT for *P. vivax*.

3.6 Haptoglobin levels

A fresh calibration curve was prepared for each kit.
Fig. 3.6 shows one of the plates with precipitation rings for the samples tested.
Table 3.6 shows the results of the tests in comparison with past history of malaria and microscopy for *Plasmodium falciparum* and *Plasmodium vivax* at the time of blood collection.
Figure 3.6a shows the distribution of the haptoglobin levels found in the samples from the 5 study areas with the mean and standard deviation for each area.
As shown in table 3.6, 49% of the samples had either hypo or ahaptoglobinaemia. 11 out of the 49 positive samples had no haptoglobin detectable at all and they were 6 from CAB (20%), 4 from IND (26.7%) and 1 from ARR (7.1%) [percentage of local samples].
Of those, 63.2% (31/49) had a history of past malaria and 44.9% (22/49) were microscopically positive for *Plasmodium falciparum* and/or for *Plasmodium vivax*. Among those who had normal level of haptoglobin, 31.4% (16/51) had a history of past malaria, but only 5.9% (3/51) were microscopically positive for *Plasmodium*. In hypo- and a-haptoglobinaemia a highly significant correlation was found with those children who had parasitaemia (X^2 = 10.86 and *p= 0.001*) while no correlation was found with malaria history. Calculating separately the correlation for the two groups hypo and ahaptoglobinaemia it was demonstrated correlation only between hypohaptoglobinaemia and positivity for either *P. falciparum* and/or *P. vivax* (X^2= 3.86 and *p= 0.05*).

3.7 Haskin determination

Three areas had urine samples tested by the Haskin method for the detection of chloroquine. SNV had 236 samples

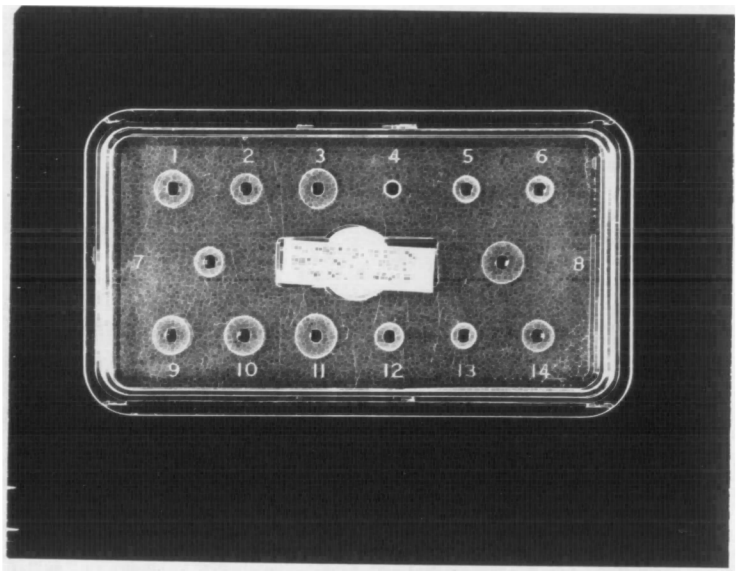


Figure 3.6

A plate of a radial immuno difusion (RID) showing the precipitated rings.

TABLE 3.6

RESULTS OF THE HAPTOGLOBIN LEVELS IN EACH STUDY AREA IN CHILDREN AGED 2-14.

STUDY AREA	SAMPLES TESTED	HAPTO L/N	MH L/N	SL L/N
SNV	20	3/17	0/0	0/0
CAB	30	17/13	11/5	11/0
PT	21	9/12	6/6	1/0
ARR	14	8/6	2/2	1/3
IND	15	12/3	12/3	9/0
TOTAL	100	49/51	31/16	22/3

L - Low level or absent

N - Normal level

PM - Past malaria history

PS - Positive slide either for *P.falciparum* or *P.vivax*

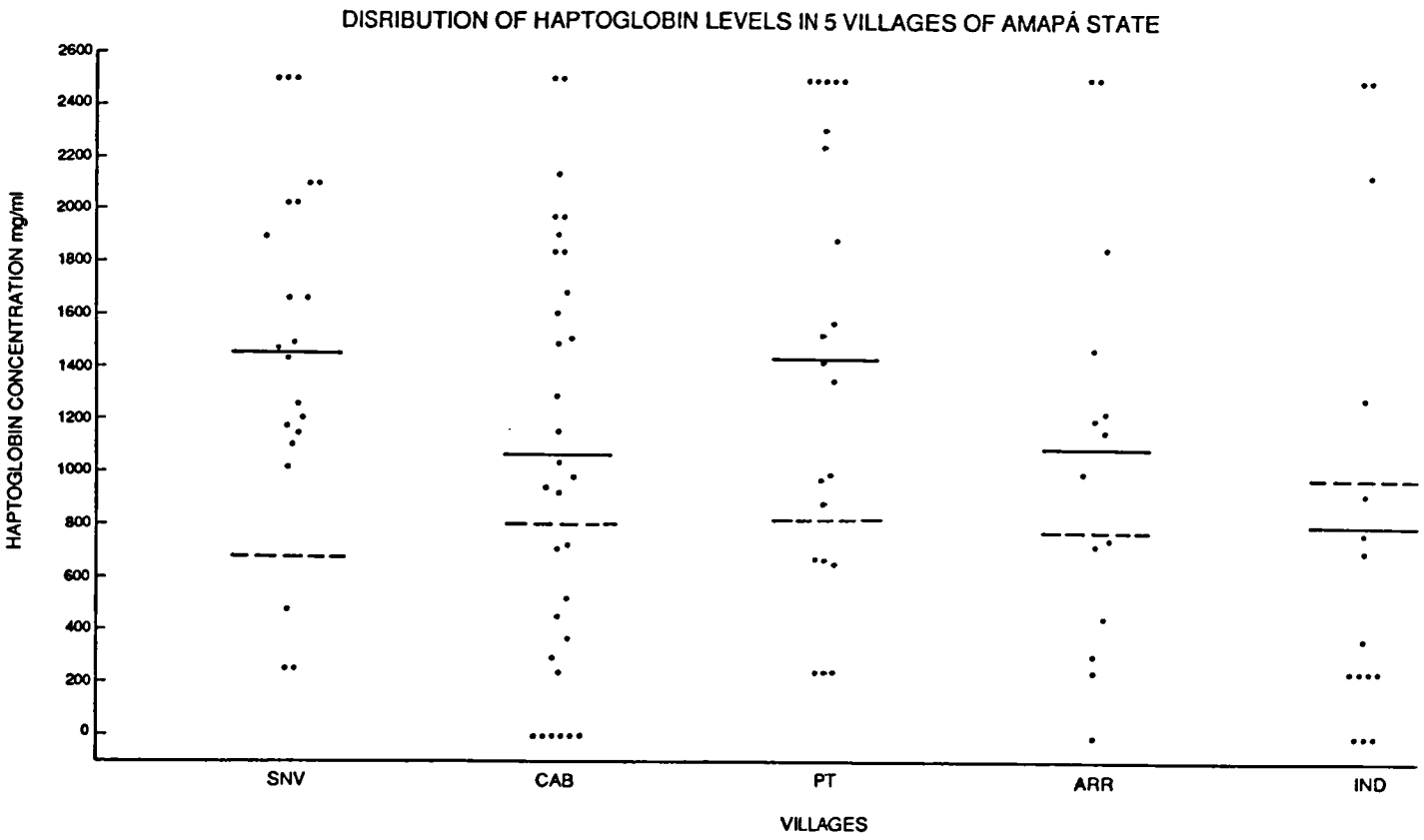


Figure 3.6a

Distribution of the haptoglobin levels of the samples from all study areas with the mean and standard deviation for each areas.

tested of which only (55.1%) were positive even though all individuals tested reported use of chloroquinised salt daily. 59.4% (63/106) of the negative samples belonged to children.
CAB had 57 samples tested and 35 (61.4%) were positive. Among the individuals with a positive result, only 13 had reported the daily intake of chloroquinised salt.
In PT where 96.9% of the sample population reported use of chloroquinised salt, 108 samples were tested and 62 (57.4%) were positive. All samples tested, positive and negative, were from individuals who had reported the regular use of chloroquinised salt.
The duration of the use of chloroquinised salt was from a few days to about 30 years for all three areas from which urine samples were tested and for all negative and positive samples (see table 3.8).

3.8 ELISA for chloroquine

As shown in Table 3.8 a total of 372 urines from SNV, CAB and PT and 626 sera from all study areas, were tested for the quantification of chloroquine. Results are shown in table 3.8.

3.8.1 Urine

370 out of 372 urine samples had a detectable amount of chloroquine. From the positive samples, 213 were from SNV, 50 from CAB and 107 from PT with a range of chloroquine concentrations of 150 to 5800 ng/ml, 23 to 7,664 ng/ml and 30 to 4,400 ng/ml for SNV, CAB and PT, respectively. All positive samples from SNV and PT were from individuals who had reported the daily intake of chloroquinised salt, while only 13 (26%) out of 50 samples from CAB had reported the same. There was no significant variation among the three study areas for the range of positivity or presence of the positive test and daily intake of chloroquinised salt. In SNV there was one sample in which 10,000 ng/ml was detected.

TABLE 3.8

RESULTS OF THE QUANTIFICATION OF CHLOROQUINE IN URINE AND SERUM BY ELISA AND COMPARISON WITH HASKIN'S METHOD.

STUDY AREA	RESULTS									CHS (%)	MEAN OF POSITIVITY	
	HK			CQUR			CQSE				CQRU ng/ml	CQSE ng/ml
	ND	N	P	ND	N	P	ND	N	P			
SNV	24	106	130	46	1	213	26	15	219	260 (100)	650	3.8
CAB	141	22	35	148	0	50	20	15	163	88 (44)	973*	5.4
PT	52	46	62	52	1	107	16	9	135	155 (96)	826	1.7
ARR	49	0	0	49	0	0	4	5	40	0	ND	2.9#
IND	27	0	0	27	0	0	2	7	18	0	ND	2.3
TOTAL	293	170	227	322	2	370	68	51	575	-	-	-

CHS - use of chloroquinised salt
HK - Haskin's method
CQUR - ELISA for chloroquine detection in urine
CQSE - ELISA fro chloroquine detection in serum
ND - not done; N - negative; P - positive
SNV - Serra do Navio; CAB - Colonia Agua Branca;
PT - Porto Terezinha; ARR - Arrependido
IND - Indigenous group
* - except one 10,000 ng/ml
# - except one 180 ng/ml

3.8.2 Serum

575 serum samples, 234 from SNV, 178 from CAB, 144 from PT, 45 from ARR and 25 from IND, were tested. As shown in table 3.8 there was some variation in the mean of serum concentration of chloroquine between the areas. CAB had the highest mean while PT had the lowest.

In SNV, the single negative sample was from a child, and all those positive had reported the use of choroquinized salt.

For CAB and PT, the areas where people had reported the in take of the salt, no correlation was found with serum positivity; CAB had a $p > 0.1$ and in PT had all negative and positive values were seen in people reporting use of the salt.

As ARR and IND areas had no report of intake of the salt, the statistical analysis was applied to those who reported the recent use of antimalarial drugs. No correlation was found in both areas between this test positivity and recent intake of antimalarials ($p > 0.8$ for ARR and $p > 0.5$ for IND).

Fig. 3.8 shows the distribution of the concentration of chloroquine detected in the urine and serum samples for those who had positive or negative slides. The means and standard deviation are also represented in the graph and no much difference was shown for the chloroquine concentration for both groups nor for urine and neither for serum samples. The T test was applied for the comparison of the means between the two groups, positive and negative slides, in urine and serum samples tested. The results did not shown significance; t= 0.76 and $p > 0.1$ (urine) and t= 1.01 and $p > 0.1$ (serum).

3.9 Distribution of mosquito species

95 daily mosquitoes captures were made during 4 trips (two each in the dry and rainy seasons). The mean of the capture time was 4 hours a day (from 6 to 10 pm). The range of temperature during all collections was 23°C to 31°C. The

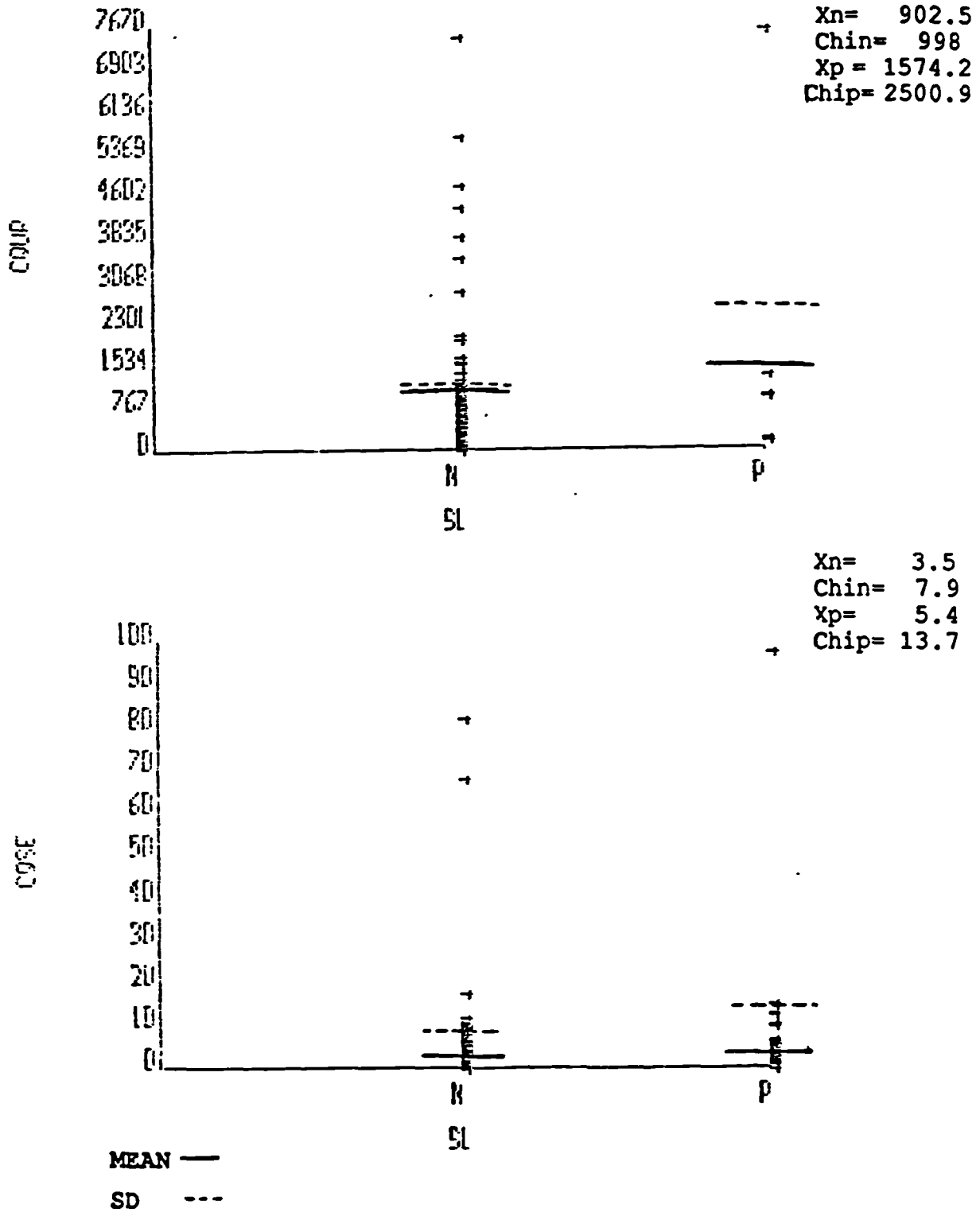


Figure 3.8

Distribution of the concentration of chloroquine detected in the urine and serum samples for individuals with negative or positive slide

relative humidity had a range of variation of 78 to 100% in the wet season and 62 - 100% in the dry and the mean was 96% and 80.3% for wet and dry seasons, respectively. However, just in a single ocasion, during the mosquitoes collection in the dry season, a rate of humidity as low as 42% was registered.

Collections were carried out during all four phases of the moon, on days with or without rain and wind. It was noted that on the rainy days or moonlight nights or on windy days the mosquito capture rate decreased.

The points for mosquitoes collection in SNV area, were those used by the ICOMI team, which are in the forest surrounding the SNV residential areas (fig. 3.9) and carpintaria. This study had also carried out captures in the forest areas along the roads (each 1 Km) that links SNV to CAB, to PT or to Macapá. The other collection points were in the residential areas of CAB, PT and ARR, where only indoors and/or outdoors captures were performed.

The number of bite/man/hour for each area during dry and rainy seasons is shown in table 3.9, and the highest index was obtained in the dry season.

Fifteen anopheline species were identified among 3053 mosquitoes collected during human biting catches in the 4 study areas. The most frequent species and their distribution by study area are shown in table 3.9a and fig. 3.9a. 96.4% of the majority of mosquitoes caught consisted of 4 species, namely, *An. albitarsis, An. braziliensis, An. nuneztovari* and *An. triannulatus*. The remainder 11 species (*An. oswaldoi, An. darlingi, An. peryassui, An. minor, An. intermedius, An. mediopunctatus, Chagasia bonneae, An. neivai, An. rangeli, An. noroestensis* and *An. argyritarsis*) were scarce.

77.2% of the total mosquitoes were collected during the dry season and their distribution by season is shown in table 3.9.

Figure 3.9 shows the the distribution of the *Anopheles* mosquitoes by places from where they were collected.

TABLE 3.9
NUMBER OF BITES/MAN/HOUR FOR THE AREAS IN WHICH MOSQUITOES WERE COLLECTED, DURING THE DRY AND RAINY SEASONS
(FEBRUARY & AUGUST 1990 AND APRIL & SEPTEMBER 1991)

STUDY AREA	DRY SEASON	RAINY SEASON
SNV	1.1	1.4
CAB	2.4	2.5
PT	4.7	1.1
ARR	14.6	4.6
CARPINTARIA*	6.5	4.4

* a carpenter's workshop and the surrounding forest which belongs to the ICOMI company and is situated at the base of the hill, on the left bank of the Amapari river facing (across the river) Porto Terezinha residential area

TABLE 3.9a

DISTRIBUTION OF *Anopheles* SPECIES COLLECTED IN STUDY AREAS (SNV, CAB, PT and ARR)

Anopheles SPECIES	SNV* W/D	CAB W/D	PT W/D	ARR W/D	TOTAL W/D	GRAND TOTAL
An. albitarsis	91/91 (43.7)	1/14 (7.0)	71/90 (48.6)	200/1407 (76.8)	363/16	1965 (64.4)
An. braziliensis	30/27 (13.7)	1/0 (0.5)	33/37 (21.1)	35/348 (18.3)	98/412	511 (16.7)
An. nuneztovari	8/41 (11.8)	61/79 (65.1)	14/31 (13.5)	6/50 (2.7)	89/201	290 (9.5)
An. triannulatus	88/16 (25.0)	7/20 (12.5)	21/16 (11.1)	7/3 (0.5)	123/54	177 (5.8)
An. oswaldoi	4/10 (3.4)	5/17 (10.2)	2/1 (0.9)	6/0 (0.3)	17/28	45 (1.5)
An. darlingi	0/6 (1.4)	0/4 (1.8)	0/1 (0.3)	1/4 (0.2)	1/15	16 (0.5)
An. peryassui	0/1 (0.2)	0/3 (1.4)	1/1 (0.6)	0/4 (<0.2)	1/9	10 (0.3)
An. minor	0/0	0/1 (0.5)	0/8 (2.4)	0/1 (<0.1)	0/10	10 (0.3)
Other species**	0/3 (0.7)	0/2 (1.0)	0/5 (1.5)	4/15 (0.9)	4/25	29 (0.9)
TOTAL	221/195	75/140	142/189	259/1832	697/2356	3053

W- wet season; D - dry season

( ) percentages determined in relation to the total number of mosquitoes collected by study area.

* includes mosquitoes collected in the "carpintaria" locality.

** other *Anopheles* species collected in small numbers included: *An. intermedius*, *An. mediopunctatus*, *An. neivai*, *An. rangeli*, *An. noroestensis*, *An. argirytarsis* and *Chagasia bonneae*.

Adult Mosquitoes

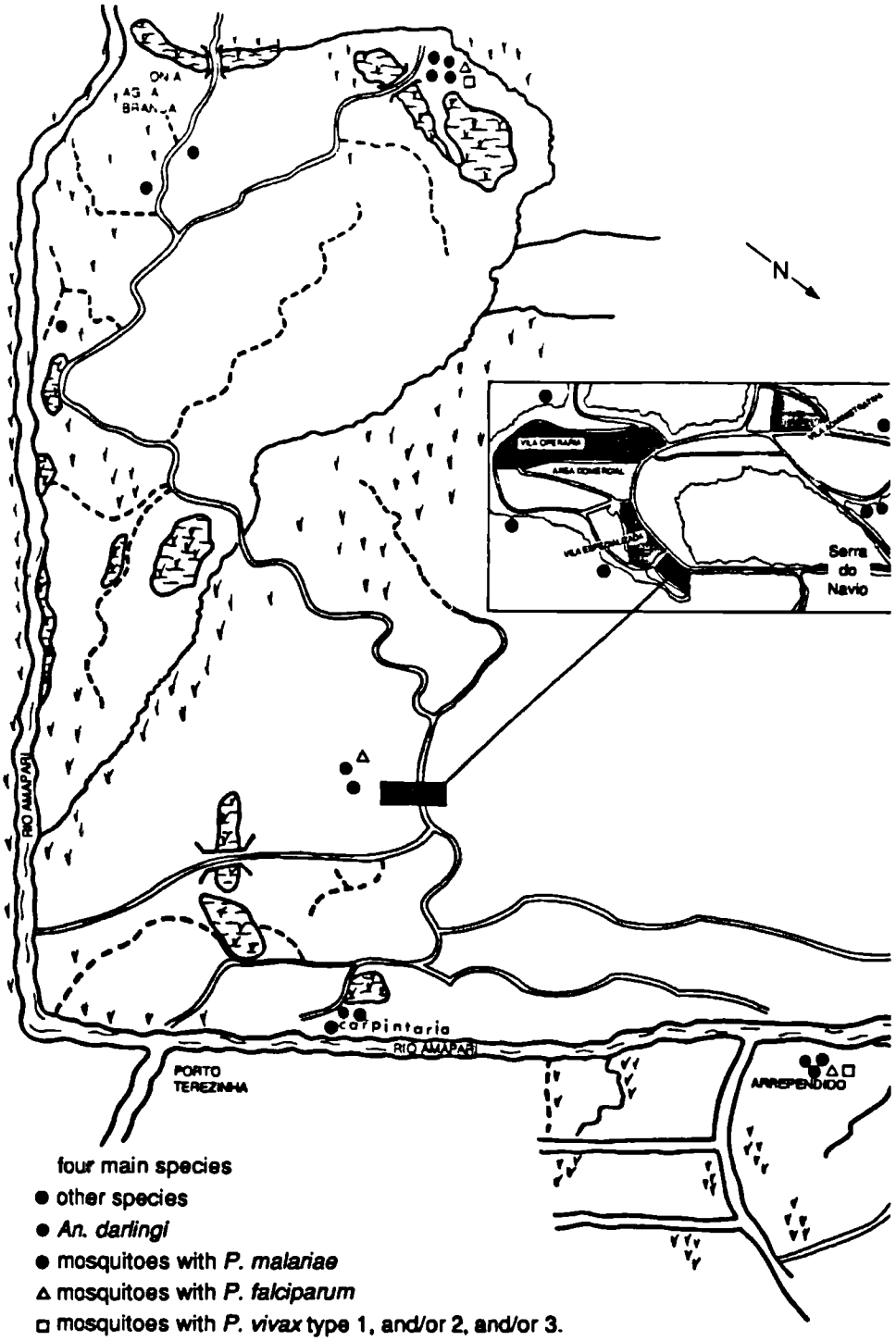


Figure 3.9

Map of SNV region with the capture points of the *Anopheles* adult mosquitoes and their distribution among the region

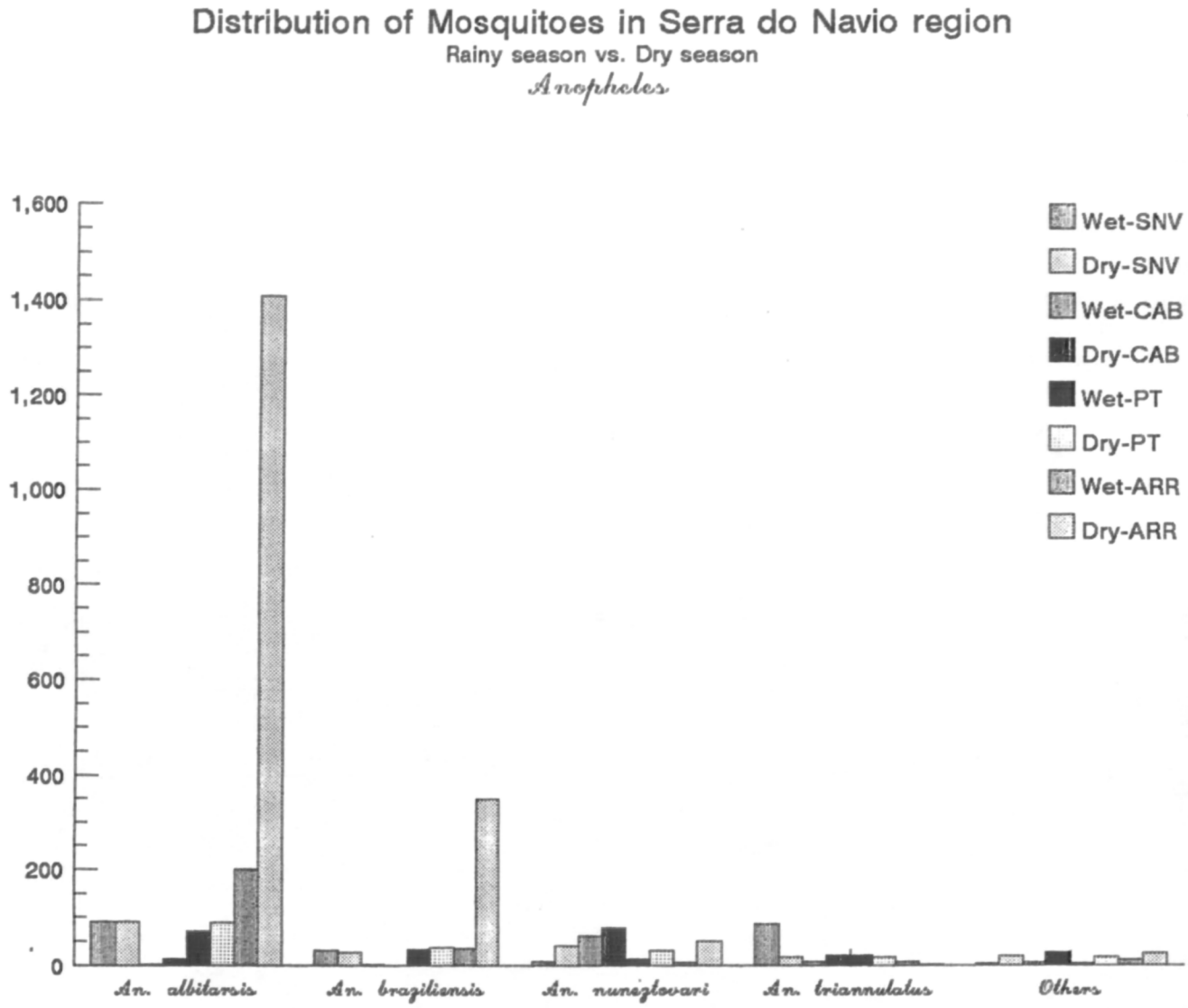

Distribution of Mosquitoes in Serra do Navio region
Rainy season vs. Dry season
Anopheles
1,600
1,400
1,200
1,000
800
600
400
200
0
Wet-SNV
Dry-SNV
Wet-CAB
Dry-CAB
Wet-PT
Dry-PT
Wet-ARR
Dry-ARR
An. albitarsis
An. braziliensis
An. nuneztovari
An. triannulatus
Others

Figure 3.9a

The data from Serra do Navio Hospital on anopheline mosquito collections during 1990 and 1991 is shown in table 3.9b. The mean number of mosquitoes collected by month is also in agreement with our catches, and the mosquito density was higher during the dry season. The species identified by the ICOMI entomological team were essentially the same as we had captured.
None new *Anopheles* species for these areas were found.

3.10 Breeding sites

Water sources and types of arboreal and epiphytic plant species belonging to the family Bromeliaceae found within all study areas were searched for the presence of immature forms of anopheline mosquitoes.
Twenty one places were positive. Their type and the areas where they were found is shown in table 3.10.
In SNV, no breeding site was found during our searches.
The water temperature and pH means for all positive breeding sites were 24°C and 5.0.
15 out of the 20 breeding sites were exposed to the sunlight and only 8 were of temporary origin formed by rainy water and/or by the invasion of river water (flooded areas). The small rivers and river edges had clear water while the other breeding sites, ponds, pools, creeks, flooded areas had not. Only the small and main rivers had weak stream.
Only 4 breeding sites were far from the residential areas (> 200m).
The vegetation is mainly on the edges of the breeding sites but also there some breeding sites such as ponds where there some small plants floating on the water.
The size of the breeding sites was variable and for ponds it was about 200 m^2. The others were much smaller and the range of size was about 20 - 50 m^2.
The distribution of breeding sites per study area is shown in fig. 3.10.
A total of 2571 immature forms of anophleline was collected

TABLE 3.9b

DATA FROM SERRA DO NAVIO HOSPITAL ON THE NUMBER OF *Anopheles* MOSQUITOES COLLECTED DURING 1990 & 1991.

MONTHS	YEAR	NUMBER OF MOSQUITOES COLLECTED	MEAN OF COLLECTED MOSQUITOES (MONTHLY)
JAN/FEB/MAR	1990	204	68
APR/MAY/JUN		146	49
JUL/AUG/SEP		359	120
OCT/NOV/DEC		550	183
JAN/FEB/MAR	1991	108	54
APR/MAY/JUN		269	90
JUL/AUG/SEP		1263	421
OCT/NOV/DEC		2141	714
TOTAL	24 MONTHS	5040	212

* all are from forest adjacent to the SNV area, and were collected by Shannon trap baited only with light.

obs: wet season generally from December to May.

TABLE 3.10

DISTRIBUTION OF POSITIVE BREEDING SITES IN THE STUDY AREAS

STUDY AREA	POSITIVE BREEDING SITES	TYPE OF POSITIVE BREEDING SITES					
		RIVER EDGE	POOLS	CREEK	FLOODED AREAS	SMALL RIVERS	POND
CAB	5	0	1	0	3	0	1
PT	4	1	0	1	1	0	1
ARR	6	2	0	0	0	2	2
ARR/PT*	4	0	0	0	1	0	3
CARP.**	1	0	0	0	1	0	0
TOTAL	20	3	1	1	6	2	7

* road that divides Arrependido from Porto Terezinha

** carpintaria - area from SNV, but it is situated at the foot of the hill facing Porto Terezinha

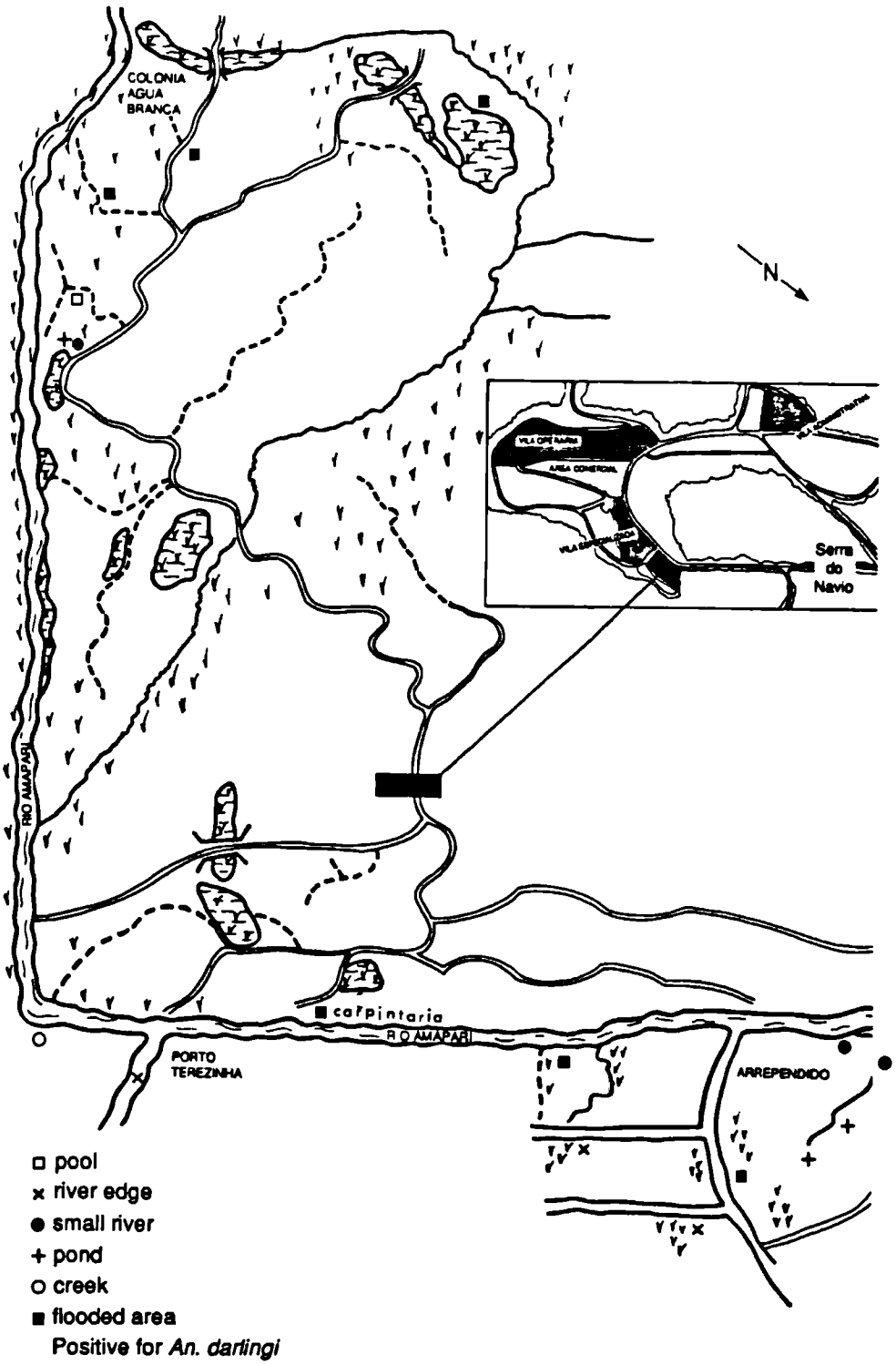


Figure 3.10

Map of SNV region with the distribution of the different types of breeding sites of the immature forms of *Anopheles* mosquitoes.

from the 20 positive breeding sites. 2013 out of the total were larvae and 558 pupae. The larvae group had 1097 specimens of 1st and 2nd instars, and 916 of 3rd and 4th instars.

The identification was carried out with 3rd and 4th instar larvae and the species found were: *An. albitarsis* (24.2%), *An. triannulatus* (38.1%), *An.braziliensis* (14.1%), *An. oswaldoi* (10.2), *An. nuneztovari* (6.5%), and others (including *An. darlingi, An. rangeli, An. intermedius* and Chagasia bonneae).

It was found only one specimen of *An. darlingi* and in a breeding site which was not the classical, i.e., clear water, shadow, small vegetation and some stream (Foratini, 1962). Rozendal, (1990) in his studies on the Guianas had also found immature forms of *An. darlingi* in breeding sites, which were not those normally described for this species.

3.11 Sporozoite rate by salivary gland dissection

Few mosquito dissections were carried out since our main objective was to know the distribution of the *Anopheles* mosquito species and if they were infected by *Plasmodium* parasites.

Mosquito dissection was a long and laborious task for our small field team. Thus, only 88 dissections were carried out. Of those 46 mosquitoes were parous, from which, 3 had sporozoite in the salivary glands (1 each of: *An. albitarsis, An. braziliensis* and *An. nuneztovari*), 4 had oocysts on the stomach wall (2 were the same specimens that had sporozoites - *An. braziliensis, An. nuneztovari;* and 2 *An. oswaldoi*). The rate of positivity for the glands was 3.4%, and for stomachs 4.5%.

3.12 ELISA with mosquitoes using anti-CS protein

The positivity rate of the total number of mosquitoes of 15 species tested by ELISA performed with CS antigen of *P. falciparum, P. vivax*-type 1, *P. vivax*-type 2 (variant VK

247 isolated in Thailand) and *P. malariae*, was 0.799% (23/2876) belonging to six species: 15 *An. albitarsis*, 4 *An. nuneztovari*, and 1 each of: *An. braziliensis*, *An. triannulatus*, *An. oswaldoi* and *An. rangeli*.
This test was performed using only the head-thorax part of the mosquitoes and individually.
9 out of 23 *Anopheles* specimens were positive for *Plasmodium malariae* (5 *An. albitarsis* and one of each: *An. nuneztovari*, *An. braziliensis*, *An. triannulatus* and *An. rangeli*); 9 were positive for *Plasmodium vivax* - variant VK 247 (7 *An. albitarsis* and 2 *An. nuneztovari*); 3 were positive for *Plasmodium falciparum* (2 *An. albitarsis* and 1 *An. oswaldoi*) and 2 for the classical *Plasmodium vivax* (1 *An. albitarsis* and 1 *An. nuneztovari*).
The positive mosquitoes either for *P. falciparum* or *P. vivax* would be repeated for the quantification of sporozoite, but as only 5 specimens (3 for *P. falciparum* and 2 for *P. vivax*) were positives it was decided to not carry out this test. However, all positive specimens had also the abdomen tested and just one (*Anopheles triannulatus*) out of the 23 mosquitoes was negative for this second test.
All mosquitoes positivve for *Plasmodium malariae* were collected in the forest adjacent to the study areas (i.e the parasites may be "*Plasmodium brasilianum*" from non human primates).

3.13 Results of the studies in another localities

3.13.1 Prevalence of mutations associated with pyrimethamine resistance

38 out of 42 (90%) of the samples studied contained the Asn-108 codon AAC which in DHFR confers pyrimethamine resistance and the remaining 4 (10%) contained only the wild-type Ser-108 codon AGC. The Thr-108 codon ACC which is linked with cycloguanil-resistant/pyrimethamine-sensitive strains, was not found within our sample.

3.13.2 Polymorphism of the CS protein antigens of *P. vivax* and *P. falciparum* in isolates from Paragominas and Jacundá cities, Pará State.

3.13.2.1 *P. vivax*

Variant forms of the human malaria parasite *P. vivax* were found in individuals from Paragominas city, since polymorphism in the repetitive and nonrepetitive regions of its sporozoite CS protein was demonstrated.

P. vivax CS protein repeat sequences from this area were the same as described from Thailand and Papua New Guinea (PNG) - ANGA(G/D)(N/D)QPG -, which differs from those described for South America, Central America and North Korea - GDRA(D/A)GQPA.

Polymorphism of *P.vivax* type 1 and type 2 CS proteins in isolates from Paragominas (Brazil) and Papua New Guinea was compared. *P.vivax* type 1 CS protein repeats were GDRA(D/A)GQPA and type 2 were ANGA(G/D)(N/D)QPG.

A distinct variability in CS sequences of clones from 4 isolates from PNG was noted and the variation found ranged from a change of a single AA residue to insertion of a stretch of AAs. In both, Brazil and PNG, isolates were foun that were a mixture of both type 1 and type 2.

Polymorphism in the nonrepeat region of *P.vivax* CS protein was also found and was restricted to three domains: amino to the conserved region in Region I, and amino and carboxyl to the conserved region in Region II.

3.13.2.2 *P. falciparum*

The two study areas, Paragominas and Jacundá, showed diversity in the immunodominant determinants of the CS protein of *P. falciparum*. Comparison of the CS epitope sequences of parasites from these two areas with Papua New Guinea with CS epitopo sequences, already identified, of parasites from Brazil and Gambia demonstrated that; 1- polymorphism was found in the Th1R-N1, Th2R and Th3R regions: AA substitutions in the Th1R-N1 and Th2R showed a conservative tendency and those found in the Th3R region

did not, and 2- the polymorphism found in high malaria-transimission areas (Papua New Guinea and Gambia) is higher than in areas from Brazil where there is a relatively low level of malaria-transmission.

3.13.2.3 *P. falciparum* ookinete vaccine antigen- Pfs25

The amino acid variation in the ookinete vaccine antigen (Pfs25), showed no variation among 14 isolates from Paragominas which had shown similar sequence to the previously described 3DT clone of *P. falciparum* isolate NF54 (Kaslow *et al.*, 1988). For the 20 isolates from Papua New Guinea one non-silent and two silent AA change were observed.

CHAPTER 4: DISCUSSION

4.1 Endemicity of malaria in the Serra do Navio region

As in Brazaville, Congo Republic (Trape, 1987), migratory movement has played an important role in the spread of malaria in Brazil (Cruz Marques, 1986), although in contrast the exodus in Brazil has been from town to rural areas and within the rural areas in the Amazon region. This exodus was first a result of a Federal governmental programme for populating and developing the Amazon region and second a result of the belief that in the Amazon region everybody would have land and it would be much easier to become prosperous. As a result, an intense migration began of farmers from the Central and Southern region, of people from all regions of Brazil for working in the building of hydroelectric plants and also the huge number of gold prospecting sites (Alecrim, 1992). At this time, deforestation and burning began in order to provide space for the population settlements, which resulted in changes in the eco-system of this region. Those changes and the growth of population settlements without adequate provision, either for basic living and/or for health-care, are thought to have contributed to the spread of malaria infection throughout the Amazon region by the formation of new breeding sites for *Anopheles* mosquitoes. The intensity of man-malaria vector contact increased which, together with the migration of human beings, helped to spread the malaria parasites from areas of transmission to areas originally free from malaria.

At this time, in the 1960's, companies such as ICOMI established their projects which created new residential localities and also new jobs, which in turn attracted more people to the area.

Even those companies that had made extensive preparation and taken precautions, faced problems of morbidity and mortality of tropical diseases, mainly malaria and leishmaniasis. This was the stimulus for ICOMI to establish

a programme for the control of malaria, the most important disease in the area. They therefore introduced such a programme which comprised measures described in section 2.1.

The migratory movement towards SNV is now not as intense because of the emergence of new places for gold prospecting attracting new migrants also exposed to malaria, where malaria is consequently the most prevalent disease.

Considering the moderate prevalence of malaria infections in the control areas and that malaria in the Amazon region of Brazil is mainly linked to occupation and migratory movement of the people (Cruz Marques, 1986 and 1987 and Alecrim, 1992), the relatively low prevalence of this disease can therefore be linked to the fact that the majority of the people work for the ICOMI company and only a small proportion of the population of these areas works either as free-lance miners or fisherman and hunters. Malaria infection in this study was due to two species of *Plasmodium* (*P. falciparum* and *P. vivax*).

SUCAM (the governmental organization for malaria programmes) had been reporting positive slides either for *Plasmodium falciparum* and *Plasmodium vivax* or for mixed infections every year in all the control areas. The prevalence recorded in this study agreed with the limited information available from SUCAM, since Amapá State is shown as an area of low level positivity for malaria parasites (WHO, 1992). Unfortunately, the SUCAM data are compiled in groups of municipalities and/or localities and generally are located under the name of the locality where SUCAM has a notification post. As a result, SNV is shown as a place of malaria transmission, but all cases reported as being from SNV are in fact from the surrounding localities which comprised the control areas of this study.

Data from the 1960s (Gusmão, 1990) show malaria infections in SNV, but also show a sharp decrease in the rate immediately after the establishment of the daily intake of chloroquinised salt as part of the malaria transmission

programme introduced first by the Federal Government and afterwards continued by the ICOMI company until today.
In SNV, where chloroquinised salt has been used daily for more than 20 years together with other methods of malaria control, and where malaria prevalence had fallen with the establishment of the programme of chloroquinised salt (Gusmão, 1990), it was not surprising to find negative slides for malaria parasites and no clinical malaria. As malaria infections had not been detected in SNV for several years, the absence of palpable spleens was expected.
This absence of malaria is supported by the Serra do Navio hospital records, where all clinical and parasitological malaria cases registered there are from areas other than SNV. During the three years of this study we did not register a parasitological or clinical malaria infection within the target area (SNV).
The endemicity levels established for the control areas were based on the population sample with positive slides and enlarged spleen. These data were collected once since the study was designed for a single sample for each control area. However, analysing the results it would have been interesting to apply a longitudinal study to the control areas to follow up the parasitaemia, the emergence of enlarged spleen and especially the immunological status of the population during different times of the year and for more than one year.
It was noted that malaria infections (positive slides) were not related to age and/or sex, and this suggests that the transmission occurs in and/or outdoors, that is, close to or within the houses.

4.2 Cultivation *in vitro*

Our inability to culture more field isolates of *P. falciparum* has been to some extent a limiting factor for this study. This is probably due to the conditions for culturing in the area; we used the Hospital facilities and laboratory, which did not provide all appropriate

conditions such as a proper sterile cabinet for culturing and an incubator with well controlled temperature. Furthermore, infected blood from the field usually contains antimalarial drugs, which interfere in the growth and development of the parasite. To avoid this interference, washing with incomplete culture medium at least twice was used on the packed blood cells. However, all of the samples were cryopreserved to provide a source of parasites from this area for any further studies.

Past experiences with field isolation of *P. falciparum* gave a similar level of failure and some measures such as purchase of a portable sterile cabinet and a small incubator with suitable temperature, were adopted in Belém to improve the results. These facilities could not, however, be taken to SNV.

Cryopreservation of blood infected samples and transfer to the Belém laboratory for recovery and cultivation was probably the most reliable method of making an isolation.For *P. vivax* the culturing process is much more complicated since it requires mainly young erythrocytes for invasion (Mons, 1990), which is a limiting factor for its continuous cultivation. Some techniques, however, such as using hepatocyte feeder cells , or using blood sources of young red blood cells (cord blood, placenta, etc.) improve efficient cultivation, but the limitations are the difficulty in obtaining these sources of blood and the cost of using hepatocytes as a routine procedure. Therefore, monkeys have also been used for the development and/or multiplication of this parasite (Mons *et al*, 1988), but as a routine procedure, it is limited due to the need for a large number of animals, a suitable animal house, and the cost of maintaining these facilities.

The discovery of new *P.vivax* variants and/or *P.vivax*-like parasites, requires their cultivation to get relevant information on their similarities (morphology of the blood forms) and/or their patterns of sensitivity to antimalarial drugs.

4.3 Drug resistance

Drug resistance has spread throughout the Brazilian Amazon region during recent years. The high level of resistance of *Plasmodium falciparum* to chloroquine in the Amazon region of Brazil (mainly Rondonia, Pará and Amapá states) has been extensively reported (Rosário , 1983, Vasconcellos *et al.*, 1983, di Santi *et al.*, 1987, Santos *et al.*, 1987). The observation of 82.5% of chloroquine resistant isolates within our sample is in agreement with observations elsewhere in the Amazon region. All chloroquine-resistant *P. falciparum* isolates were found in the control areas. The spread of chloroquine resistance is thought to be mainly due to the extensive use of this antimalarial drug as the first choice for treatment of falciparum malaria and also because of its inappropriate use (Wernsdorfer & Payne, 1991). There is also a belief that the daily intake of chloroquinised salt as a malaria transmission control programme introduced in Brazil during the 1950's (Pinotti, 1953 and 1955) contributed to the spread of chloroquine resistance (Payne, 1988). There is no proof, however, of this hypothesis. The results here show no correlation between the presence of chloroquine resistance and the personal use of chloroquinised salt. Since it is now many years since the introduction of protection using treated salt, it is unlikely that such a comparison will tell us whether salt was relevant to the appearance of resistance in the late 1950s and 1960s, and the sample (17) is far too small to detect anything but a very obvious correlation. Amodiaquine resistance, which was less common than chloroquine resistance in our sample has been reported previously in Brazil. Some amodiaquine resistance in areas of high level chloroquine resistance can be due to cross-resistance between the 2 drugs and not only due directly to use of amodiaquine. In Brazil and in our study area amodiaquine has been used for the treatment of falciparum malaria and thus this may be the reason for the level of resistance to amodiaquine found among the samples.

In the 1950's, quinine, though effective against falciparum malaria was considered to be too toxic for routine therapeutic use (Findlay, 1951) and its use was discouraged: chloroquine, which was the newest and most efficient antimalarial drug, became the drug of choice for the treatment of falciparum malaria. The emergence of the resistance of *P. falciparum* to chloroquine, and the consequent need for alternative drugs for the treatment of chlorquine resistant falciparum malaria, has led to the reintroduction of quinine, alone or in association with tetracycline.

Mefloquine, is a drug developed in the 1970s, which was largely used in Thailand where *P. falciparum* is showing resistance (Wernsdorfer *et al.*, 1991). Based on these data on the rapid emergence of resistance, the use of mefloquine for treating malaria in Brazil was more carefully applied and evaluated. In Brazil, mefloquine is used mainly in those cases where the infection does not respond to any other treatment or for very high parasitaemia which can produce a more severe malaria. It is also well known that there is an illegal market for mefloquine, mainly among the miners (Souza, 1992). There are no clear reports, however, of resistance of *P. falciparum* to this drug in Brazil. Although Souza, (1992) comments on a possible few cases reported as mefloquine resistant, he also stated that better evaluation had to be carried out on similar cases in order to determine if it was a true resistance or a matter of inadequate cure due to early vomiting, diarrhoea or intestinal malabsorption. In the experience of our team at the Evandro Chagas Institute, where hundreds of falciparum infected blood samples have been tested, no resistance to mefloquine *in vitro* has been demonstrated.

Our results show that quinine and mefloquine should still be effective in our study areas for treatment of falciparum malaria.

Concerning *P.vivax* malaria there are no confirmed reports about this parasite's resistance to antimalarial drugs in

Brazil. The treatment adopted for vivax malaria, in Brazil, is chloroquine for the blood forms together with primaquine for the tissue forms in order to avoid relapse. Garavelli & Corti, (1992) reported a presumed case of chloroquine resistant *P. vivax* in Brazil, but this was extensively queried by other investigators (correpondence, *Trans. Roy. Soc. Trop. Med. Hyg.*, 86, 570-571).

Most samples tested for sensitivity to antimalarial drugs were from CAB but this was due to chance, since we collected blood samples from everyone coming to the SNV hospital and/or SUCAM post who was positive for malaria parasites.

We also carried out drug tests on samples from individuals who did not live in the control areas. Although these were not included in the results, it is relevant to note that they were mainly from mining areas and that the majority of their falciparum strains were chloroquine resistant. Amodiaquine and a few cases of quinine resistance were also detected.

Although this study did not investigate pyrimethamine resistance in this area, a PCR study showed the wide spread of this resistance throughout the Amazon region (Peterson *et al.*, 1991), and this drug has failed in the treatment of malaria in Brazil (Almeida Netto *et al.*, 1972). Pyrimethamine resistance is probably widely distributed as chloroquine resistance in the study area. This needs to be confirmed by further studies.

4.4 Immunological status of the population

The results obtained from the immunological techniques, in particular the IFAT tests show that the population has been exposed to malaria infection. Seropositivity and prevalence of high titres were age related, except for in the indigenous area where all titres were represented in all age groups. In SNV there was one individual (9 years old) who had a titre of 1:160 for *P. falciparum* by IFAT (blood stages) and no positivity either for IFAT with *P. vivax* or

for ELISA with *P. falciparum* blood stages. No explanation for this finding was found, since there was no history of malaria and/or any other disease that could have produced a cross reaction. In PT, there were 3 cases of children (2 of 3 years old and one of 4) with high titres by falciparum IFAT of which 2 were seropositive by vivax IFAT. These 2 children had had malaria more than once. The other child had no positive test by vivax IFAT, no history of malaria and no present infection, which, as for the child from SNV, could not be explained.

In SNV the correlation between IFAT positivity (blood stages) and malaria history was demonstrated. Those with an history malaria were reported to have had malaria more than 10 years before. This suggests that their antibodies persist for a long period of time. Despite correlation for the control areas between IFAT seropositivity and past malaria, it is not possible to determine the longevity of their antibody reactivity because they had several past malaria infections within different time periods.

Statistical analysis was applied to assess the correlation between the positivity of the IFAT test and other variables (MH, SR or SL). This analysis was carried out for each area separately and all control areas taken together and the highest correlation was obtained from the analysis of all control areas together. The IFAT for either *P. falciparum* or *P. vivax* demonstrated correlation with all three variables in all age groups, except in the adult group for *P. falciparum* IFAT and positive slide (SL) and for *P. vivax* IFAT (IV) and history of malaria (MH). The lack of such correlations could be due to, in the case of IFAT for *P. falciparum* vs SL, the fact that these adults have already been infected several times by *P. falciparum* so that a fresh infection did not lead to a rise in rate of IFAT positivity; and in the case of IV vs MH, the fact that past malaria in all areas is strongly linked to past falciparum infection rather than to vivax infection.

In addition, the correlation among the combined children

was much stronger when the analysis was performed for individual locations. These correlations demonstrate, that it is best to examine children to understand the immunological status and/or the pattern of malaria transmission within a certain population, for example if spleen rate and parasite rate in this group are to be used to determine endemicity in an area.

The results from the ELISA tests are intriguing since for SNV, CAB and PT the rate of positivity was much higher than for IFA tests while in ARR it was very low. As the control areas are similar and had given a similar pattern of antibody response by IFAT the question of the sensitivity and/or specificity of the ELISA arises, since if the test was highly specific the results would not be so high in SNV and in PT, but much higher in the ARR and IND areas. If it was highly sensitive, the results for at least the IND areas would also be higher.

It is possible that this ELISA test using whole-parasite antigen, is not yet completely standardised worldwide for epidemiological studies or for diagnosis. To support this hypothesis, the indigenous area, where malaria transmission occurs frequently among all age groups of the population, had a lower level of positivity with ELISA when compared to IFAT (33.3% and 100% respectively).

Using the rate of infection obtained from IFA tests by age group for all study areas and analysing them with the method of Draper *et al.*, (1972), it was demonstrated that the risk of one person being infected within one year is less than 1% for SNV and less than 5% for the control areas. These findings are in agreement with the difference in prevalence of malaria infection established in this study for the SNV and PT study areas. CAB and ARR were classified as mesoendemic areas for malaria transmission (parasite rate in children between 10 and 25%), on the basis of IFAT results, but according to the ELISA data alone these areas could not be classified satisfactorily. Discussion on the most sensitive and/or specific

immunological method for diagnosis with Dr. David Warhurst (personal communication) led to the conclusion that IFAT is still the best method for detecting antibody in malaria. The low level of false positivity, which is nevertheless always lower than with other methods.This study did not examine innate immunity and related factors since the number of young children sampled was in fact very low. There were few pregnant women with malaria in all areas (data from the Hospital - less than 5%) and there were no data available concerning congenital factors such as G-6-PD deficiency, thalassaemias or sickle cell disease for the Amazon region, younger children were not necessarily expected to show innate immunity.

Other studies on the immunological response to malaria infection in Acre and Pará states of Brazil (Kremsner, 1992 and Arruda *et al.*, 1989) found similar rates of seropositivity within their target populations.

The studies on antibody to malaria CS indicate that in all areas the population had experienced contact with malaria sporozoites through the bite of infected *Anopheles*. Antibody prevalence for *P.falciparum*, *P.vivax* type 1 and *P. malariae* CS have already been reported from other areas of Brazil (Arruda *et al.*, 1986 and 1989, Kremsner *et al.*, 1992). The existence of the variants of *P.vivax* (type 2 and type 3) in the study was not known, although type 2 had been reported from areas in Acre State (Amazon region) by Kremsner *et al.*, (1992) using ELISA and by Qari *et al.*, **(in press)** by sequencing the CS protein gene. Type 3, the most unexpected, has recently been discovered using PCR amplification and sequencing of the CS gene (repetitive sequence: APGANQEGGAA) with human "*P.vivax*" infections from PNG and Brazil (Qari *et al.*, **in press**). This parasite is closely related to *P.simiovale*, which is a parasite of old world monkeys (Garnham, 1966). However, anti- *P.vivax* type 2 and 3 sporozoite antibodies have been demonstrated here in the populations of all areas. Both types are now believed to be widespread in Brazil since there are reports

of the existence of type 2 and 3 from Paragominas and Jacundá cities in Pará State (Qari *et al.*, **in press**), type 2 from Acre State and, reported here, type 2 and 3 from Amapá State. Although all three states are in the Amazon region they are very distant from each other. Malaria infection caused by "*P. ovale*" has been registered in PNG, but since PNG has no monkeys and Brazil has no Old World monkeys, the origin of this *P. simiovale* -like parasite malaria in Brazil and PNG needs further investigation.

The inability to demonstrate any correlation between the positivity of ELISA using CS antigen and reported intake of chloroquinised salt in CAB, is not entirely unexpected since the majority of reported malaria infections and/or positive slides for malaria parasites concern *P. falciparum*, which in our study have shown a high level of resistance to chloroquine (more than 80%). It is probably, therefore, that the individuals with positivity for ELISA using CS antigen who had recent malaria infection caused by *P. falciparum* and reported the intake of chloroquinised salt, were infected with a chloroquine resistant strain.

When the same correlation (positivity for ELISA using CS antigens vs reported intake of chloroquinised salt) was performed for CAB and PT together, a significant correlation was demonstrated, i.e., there were significantly more person with negative slides in the group taking chloroquinised salt and showing positive CS antibodies, than in the corresponding group not taken the salt. This fact is mainly because in PT there were few individuals with positive slides (prevalence 6.25%), all of whom had reported the use of treated salt.

In SNV, where all the population sample reported the regular use of chloroquinised salt and where no positive slides for malaria parasites were detected during our study, statistical analysis could not be carried out. A proportion of this population had shown antibodies against CS antigen, which implies that they had been bitten by an infected *Anopheles* but did not develop the disease

clinically and/or parasitologically because unlike some antibodies CS antibodies are known be short lived. It is possible to suggest then, that they had been protected by the action of chloroquinised salt since this is the only control measure which targets the parasite that is adopted by those in SNV.
Considering that, at least, two whole SNV families included in this study, were positive for one or more malaria CS antigens tested and that no *Anopheles* mosquitoes were detected inside their houses, the most likely suggestion is that they were bitten in the forest area that surrounds the SNV residential and industrial areas, where they usually go for leisure and where, we know from this study, that there are infected *Anopheles*.

4.5 Haptoglobin: an indicator of malaria infection?
For Brazil, there are no data available in the literature available on haptoglobin levels in areas where malaria transmission occurs, while for other countries in Africa there are several reports showing that the hypo and/or ahaptoglobinaemia are strongly linked with malaria infection. Our results from areas of light and medium level malaria transmission areas now clearly show a significant correlation between low level and/or the absence of haptoglobin and malaria. In SNV a non-endemic area, the sample tested had displayed normal and high levels of haptoglobin.
Surprisingly, a correlation between ahaptoglobinaemia (absence of haptoglobin) and malaria was not seen whereas it was seen for hypohaptoglobinaemia and malaria. It was expected that the correlation with ahaptoglobinaemia would be strongest, as has been shown in children from Africa. The number of children without haptoglobin in their sera (11 out of the 49 with abnormal levels of haptoglobin) was, however, insufficient to show a correlation. Nevertheless, considering the analysis for the entire group of children who had an abnormally low level of haptoglobin, it is

possible to conclude that as in Africa, in Brazil haptoglobin can be used as an indicator of malaria infection.

4.6 Chloroquine in urine and serum

The results obtained from Haskin's method and ELISA for the detection of chloroquine in urine and serum samples confirmed the high sensitivity of the ELISA test in comparison with the HK colorimetric test (Shenton *et al.*, 1988), and the difficulty of achieving a steady level of chloroquine in human fluids sufficient to protect against malaria infection. The sensitivity of the Haskin test demonstrated by Shenton *et al*, 1988 was 3 µg/ml and in the comparison between this test and ELISA, showed that Haskin failed to detect 30% of the urines positive detected in ELISA. In our study the failure of Haskin test was about 50% and the ELISA test detected chloroquine concentrations as low as 1 ng/ml. The negative results of the Haskin's test for some urines with positive ELISA test is not unexpected. The serum level of chloroquine recognised as protective against malaria was established by WHO, (1973) as an average level of 30 ng/ml. Salt intake varies from individual to individual and is low in young children who normally have more sweet and/or lightly salted meals, in adults who have an unsalted diet due to medical advice, and in those who use lightly salted food because they do not enjoy the taste. In addition, the number of salted meals/day and the excretion rate of chloroquine varies individually (Paulini & Pereira, 1963), and can affect the detection of chloroquine. Thus, the range of amounts of chloroquine detected in both urine and/or serum samples can be very variable individually and within a population. This does not mean that the amount detected is or is not protective.

The mean concentration of chloroquine detected in urine showed variation between areas. For serum this variation was also observed but was larger.

Paulini & Pereira, (1963) carried out studies on the renal excretion of chloroquine derivatives and found, that mean rates of individual excretion differed statistically.
The results of high variability obtained in the individual ELISA tests for chloroquine concentration in urine has support in the study described above. A plot of distribution of urine or serum chloroquine concentrations versus the current malaria infection is shown in fig. 3.8 and no significant difference in levels between the two groups was seen.
An analysis of the mean and standard deviations of the concentration of chloroquine detected in both urine and serum in individuals with either positive or negative slides for malaria parasites was performed (t-test), no significant difference was found between the mean values either for urine and or for serum ($p > 0.1$ for both). There was no evidence from this that chloroquine in the serum or urine indicated any protection against infection.

4.7 Epidemiology of malaria transmission in the Serra do Navio region

The previous data from SUCAM, shows only that malaria infections in the SNV region are mainly caused by *P. falciparum* and *P. vivax* and rarely by *P.malariae*.
As shown by the results of this study the *P. vivax*-type 2 (variant VK 247) is also widespread in this area since within our sample populations antibodies against the specific CS antigen of this variant were detected and the sporozoite of this variant was identified in mosquitoes. Whether the clinical features, incubation and patent period, relapses and the response to antimalarial drugs is similar or not to those described for the classical and well known *P. vivax* needs to be investigated.
The new "*P. vivax*-like" parasite (type 3), which was detected in the population was not tested for *Anopheles* mosquitoes. As it has been reported to be circulating within two other populations of the Amazon region

(Paragominas and Jacundá in Pará state), we believe that like the other types of *P. vivax*, it is widespread in the Brazilian Amazon. As for variant VK 247, nothing is known about the clinical features, relapses, or sensitivity to antimalarial drugs.

There were no previous published data about *Anopheles* mosquitoes from this area and no study of transmission has been carried out in this region by SUCAM. There are records, however, of the *Anopheles* species distribution in the SNV area obtained by the malaria control programme of the ICOMI company. These reports show that in the 1960's *An. darlingi* was found even in some places close to the SNV residential areas. Due to the introduction of the control programme, this species had disappeared from SNV and has only been reported, occasionally, from the "carpintaria". As *An. darlingi* is very anthropophilic (Deane *et al.*, 1948), the introduction of control measures against the vector caused this species of *Anopheles* to lose its main source of blood (man) and this probably led to the failure of the population to survive in or close to SNV itself.

Our findings support the data from ICOMI since we captured a small number of *An. darlingi* (16 specimens)only from the carpintaria, CAB and ARR but none from SNV and PT. Its distribution is basically in the areas localised at the foot of the hill. On the top of the hill, where the SNV residential and administrative areas are settled, the *Anopheles* species captured during our study were species known to be more zoophilic than anthropophilic. In the forested areas along the roads the species captured were the same as those captured on the top of the hill, with only slight changes in the density and diversity of the *Anopheles* mosquitoes when the collections were performed close to the residential areas. Thus, those species captured in the forest areas are mainly zoophilic and their maintenance is supported by blood meals from animals other than man.

Reports on malaria vectors in Brazil are few. Based on

studies carried out on the 1940's and 1950's by Prof. Deane's team, it was established that along the coast, malaria was transmitted with a low level of endemicity by the salt-water breeder *An. aquasalis* and in all other areas where malaria transmission occurred, *An. darlingi* was the vector. They also demonstrated, however, that some other species such as *An. albitarsis* and *An. braziliensis* could be of secondary importance as malaria vectors.

Only in the 1980's Arruda *et al.*, (1986) carried out studies on the distribution of *Anopheles* species, and on their role in malaria transmission in Pará state and found other *Anopheles* species carrying malaria sporozoites by ELISA and RIA (radioimmunoassay) and suggested they are possible malaria vectors. The species are: *An. darlingi, An. albitarsis, An. oswaldoi, An. nuneztovari* and *An. triannulatus*. The last three had not been found infected in Brazil. All species harboured *P.vivax*, but *An. darlingi* and *An. oswaldoi* also harboured *P. falciparum*. Tadei *et al.*, (1988), studied the distribution and the dynamics of malaria transmission in Ariquemes in Rondonia State. They demonstrated by positivity rate for sporozoites in the salivary glands using traditional dissection that *An. darlingi* is the main vector in this area, while by ELISA they reported positivity in *An. nuneztovari, An. triannulatus, An. galvoi* and *An. peryassui* for *P. vivax*, and double positivity for, *P.falciparum* and *P. vivax*, in *An. darlingi*. Klein *et al.*, (1991) pointed out in their study the role of *An. albitarsis* as a malaria vector in Rondonia State. In the present study, in an area far from all previously reported, we have found 15 different species of *Anopheles* mosquitoes and also found other *Anopheles* than *An. darlingi* carrying human malaria sporozoites detectable by ELISA. The species were the same as those found by Arruda and colleagues, plus *An. braziliensis* and *An. rangeli*. The latter was positive only for *P. malariae*.

Despite the apparently low rate of positivity of the

Anopheles mosquitoes for malaria sporozoites, either detected by salivary glands dissection or by ELISA, it is well known that this low positivity is more than enough to maintain continuity of malaria transmission, nevertheless the finding of an *Anopheles* mosquito infected with a malaria sporozoite does not mean that it is a vector (Wirtz *et al.*,1988). To incriminate an *Anopheles* species as a vector, studies on the behaviour and habits of the species (anthropophilism, possible endophilism, time of biting, life expectancy, life expectancy of infected females, vectorial capacity, etc.) have to be carried out. The positivity for the sporozoite, at least, demonstrates that the species can be infected with malaria parasites, which classifies it as a candidate vector.

Since all *Anopheles* species were collected with human bait, they are obviously sufficiently anthropophilic to be malaria vectors.

As *An. albitarsis* was caught only with human bait, presented the highest density over all study areas, and was positive by ELISA and by salivary gland dissection for malaria sporozoites, it probably plays an important role in the epidemiology of malaria in these area. Nonetheless, it is worth noting the number of *An. nuneztovari* caught in CAB, which as in Venezuela (Galbadon, 1981), in Suriname (Rozendal, 1990) and in other areas of Brazil probably plays a role in malaria transmission.All *Anopheles* mosquitoes positive for *P. malariae* were collected in the forested areas, where on several occasions during the catch we saw small monkeys. As *P. brasilianum* (monkey malaria parasite) is genetically and morphologically indistinguishable, it is possible that a sylvatic cycle of this parasite is taking place in such areas. Our records did not show infection in man caused by *P.malariae* and the Hospital records and SUCAM data shows a very low prevalence of this parasite, and yet the highest positivity for *Plasmodium* in mosquitoes was for this species. The maintenance of this *Plasmodium* could therefore be through

the monkeys.
The positive breeding sites had the immature forms of those *Anopheles* species that were also captured as adults, demonstrating that these breeding sites are important in the maintenance of all these species.

4.8 The role of chloroquinised salt as a preventive measure for malaria in the study areas.

In all study areas, at least, one control measure for preventing malaria has been applied, that is the house-spraying of DDT every 6 months, which for the past years has been applied irregularly by SUCAM. This is all that has been done for ARR and IND, while in PT and CAB a certain proportion of the population also use individual protection such as bednets and/or domestic insecticide.
Resistance of the *Anopheles* mosquitoes to DDT has been reported from different areas where malaria transmission occurs. Suarez *et al.*, (1990) reported resistance of *An. darlingi* from an area of the pacific coast of Colombia, and Tauil, (1992) refers to insecticide resistance in mosquitoes from some areas of Brazil. Thus, the continuity of malaria transmission in Brazil, where DDT has been applied needs further investigation in order to determine the spread and intensity of insecticide resistance.
In PT the majority use chloroquinised salt as a preventive measure while in CAB a smaller proportion of the population used this salt.
To establish some correlation between the habit of daily intake of chloroquinised salt and protection against malaria infection, we measured chloroquine in the urine and serum, measured the positivity rate for *Plasmodium* infection, and/or the level of anti-sporozoite antibodies. Different factors may influence the results, such as other measures for controlling malaria transmission. Nevertheless, in SNV where other control measures were applied, mainly to avoid the man-vector contact inside the residential area, and where no malaria had been reported,

measurement of the level of antibodies to sporozoites may be a possible mechanism for examining correlation between intake of the salt and protection against malaria infection.

As is well known chloroquine, a blood schizontocide, prevents the development and growth of malaria parasites in the blood stream. Consequently, the release of merozoites into the blood for the reinvasion of erythrocytes does not occur and a new blood cycle does not take place.

Other control measures such as: treatment and/or destruction of breeding sites, spraying of DDT, screened windows and doors, good sanitation for all residential and industrial areas, the existing cordon sanitaire separating the populated areas from the forest areas, have been used in SNV to decrease and/or stop man-vector contact. These should not affect the relationship between positive blood slide and anti-CS antibody.

Of course, the results obtained in SNV where a dramatic decrease in malaria was reported in the 1960's and thereafter no malaria infection has been reported within the population of SNV, is due to the use of a combination of control measures.

The results of this study show, however, that about 40% of the sample of the SNV population studied have specific antibodies against CS antigen but have not developed infection, clinically and/or parasitologically despite their contact with infected *Anopheles* mosquitoes. It is arguable therefore that they have been protected by the only control measure which targets the parasite, chloroquinised salt.

The SNV population has been protected against *P. falciparum* despite the high level of chloroquine-resistant *P. falciparum* strains in the control areas. One explanation would be that the strains of *P. falciparum* circulating in the area of SNV are chloroquine sensitive.

The results do not demonstrate whether the anopheline population from Serra do Navio is isolated or not from

those found in the control areas. The probability of interchange between the populations is high, since the distance between SNV and the control areas, especially CAB is not great (Eyles, 1944). Also the ability of mosquitoes to fly long distances and be carried by wind (Eyles, 1944), are factors that support the idea that the anopheline populations are not separated. The lack of infected people in SNV supports the idea that mosquitoes became infected by blood meals taken from people in the surrounding areas where it was known that chloroquine resistant *P. falciparum* strains circulated. Why then was there no infection in SNV if the residents were bitten by infected *Anopheles* as demonstrated by the detection of anti-sporozoite antibodies? *P.vivax, P. malariae* and probably the variants of *P.vivax* are chloroquine sensitive, the intake of chloroquinised salt probably protected against the infection. In the case of *P. falciparum*, although the number of people presenting anti-falciparum CS antibodies was not high (about 10% of the population sample) and they were detected in more adults than children (16 aged 5 to 13), it is still difficult to explain the complete lack of infection by *P. falciparum*. As suggested above it seems that by chance the infected mosquitoes that had bitten these individuals were infected only with the falciparum chloroquine sensitive strains. A possible explanation is that *An. darlingi* transmits the resistant strains and is not found near SNV, whilst those mosquitoes in SNV only transmit chloroquine sensitive *P. falciparum* (Sucharit *et al.*, 1977, Wilkinson *et al.*,1976). The intake of other antimalarial drugs could be an additional reason for suppression of the infections, but there is no evidence of this intake from the questionnaire in this study. Although the absence of *An. darlingi* is suggestive, why *P. falciparum* infection is not found in SNV remains a mystery.

4.9 An overview of malaria control in Brazil

The control measures adopted by the Brazilian government

for malaria, based on WHO's malaria eradication programme have been based on the assumption that: 1- transmission occurs predominantly indoors, 2- there is no animal reservoir of epidemiological importance and, 3 - insecticide applied to surfaces provides contact and residual effect (Tauil, 1992).

This programme has failed mainly in the Brazilian Amazon where the increase of malaria was due to the migratory movement, the emergence of drug resistance and the failure of the effect of the control measures applied to target the *Anopheles* mosquito.

Cruz Marques, (1986) and (1987) carrying out an analysis of the spread of malaria in Brazil showed that such spread started in the 1960's with the building of new roads linking the south with north, continued in the 1970's with the establishment of hydroelectric projects and in the 1980's with the emergence of the gold prospecting sites. He classified the old transmission areas in the Amazon region in Brazil as "stable malaria", and areas where transmission had recently be introduced as "frontier malaria". The latter, characterized by high vectorial density; exposure to vectors and extradomiciliary transmission; low level of immunity in the migrants; high morbidity but relatively low mortality; a high proportion of cases of *P.falciparum;* difficulty in the application of conventional control measures and high population mobility.

Due to all the changes observed in malaria transmission within the Amazon region summarised above, the Brazilian governmental malaria control programme is redirecting plans. The new strategies consider the characteristics of malaria transmission one by one in the different epidemiological zones, that is the new control strategy is the identification of main local risk factors and the subsequent adoption of appropriate measures. For example, in the human settlements in gold prospecting areas the houses have no wall, so, what is the objective in spraying these "houses"?

For areas such as gold prospecting sites it is very difficult to establish which control measures will be effective since house-spraying is useless, the antimalarial drugs are ineffective due to the high level of resistance and the miners are highly exposed due to the duties of the job. So, the long term hope would be a vaccine that is not usually influenced by the socio-economic status of the population. The trials with vaccines have given rise only to doubts due to the lack of proven efficacy.

A vaccine trial is being carried out in Rondonia State in Brazil, using the blood-stage form vaccine (three fragments of antigens from the merozoite linked by a repeating peptide of the CS protein) produced by Dr. Patarroyo but no preliminary results have been reported. This vaccine is also under evaluation in a double-blind randomized placebo-controlled study that has started in Tanzania in January of this year, since the data from the trial in Latin America was inconclusive (TDR news, 1992). Other candidate antigens for vaccines are being investigated and an effective new tool for controlling malaria may result.

The Meeting of WHO held in Amsterdam last year resulted in a global malaria control strategy which suggested that each country based on its specific characteristics of malaria transmission had to identify local problems and priorities in order to apply appropriate interventions (WHO, 1992c). The basic technical elements of the strategy have to be: early diagnosis and prompt treatment, planning and implementation of selective and consistent preventive measures, early detection, containment or prevention of epidemics and, to be up to date with the malaria situation in the country, especially for the determination of ecological, social and economical factors relevant to the transmission of the disease.

Based on the suggestions described in the last paragraph and considering the classification made by Cruz Marques, (1986) and (1987), the control areas of our study, CAB, PT and ARR have the characteristics of areas of "stable

malaria". These characteristics together with the facts that the houses are walled, the transmission is intra and/or peridomiciliary and the prevalence of malaria parasites is not so high, we believe that the identification of the risk factors of these areas will provide some useful tools for the design of an effective plan for malaria control. Suggestions for appropriate control strategies are listed in the section on recommendations.

CONCLUSIONS

The epidemiological picture of malaria infection in all study areas has been well established:

1 - The malaria infections are mainly caused by *Plasmodium falciparum* and by *Plasmodium vivax*: "2 additional variants" of *P. vivax* have been found circulating within the populations;
2 - All study areas, except IND are areas of hypo to meso-level of endemicity of malaria;
3 - Malaria infection in all areas other than SNV, is not related to sex and/or age;
4 - In the control and IND areas, malaria is associated with the presence of enlarged spleen;
5 - In CAB and PT, the intake of chloroquinised salt is not related to the prevalence of the infection;
6 - Chloroquine and amodiaquine resistance occur in all control areas.
7 - Quinine and mefloquine should still be effective as antimalarial drugs for treating falciparum malaria in the control areas, according to *in vitro* results.
8 - Seropositivity agaisnt malaria parasites is seen to be age-related in the population studied;
9 - In SNV, CAB and ARR, the IFAT-determined seropositivity against *P. falciparum* is associated with history of malaria and is seen mainly in the adults;
10- All individuals samples from IND show IFAT antibody against *P. falciparum*;
11- In all studied areas, the antibody prevalence against *P. vivax* is lower than that against *P. falciparum*, but for ARR this difference is very small;
12- In SNV and ARR, the presence of antibody against *P. vivax* is not related to malaria history;
13- The presence of antibody against *P. vivax* in CAB and PT is related to malaria history especially when the whole group (children and adults) is taken together, but also when children are considered separately;

14- The risk of becoming infected with malaria parasites is low (1-5% per year) for all control areas but lower (< 1%) for SNV;

15- The ELISA test using asexual blood stages of malaria parasites as carried oput in this study, has given unsatisfactory results;

16- ELISA using malaria sporozoite repetitive CS antigens has been valuable for demonstrating contact between people and infected mosquitoes;

17- In all studied areas individuals were found who had been challenged with more than one species or type of malaria sporozoite;

18- The low level of haptoglobin in children is an indicator of malaria infection in control areas and IND areas;

19- Despite the low sensitivity shown by Haskin's method when compared with the ELISA test for detecting chloroquine, this method is applicable to the purpose of monitoring the daily intake of chloroquinised salt;

20- ELISA for chloroquine detection is also a good tool for monitoring the intake of chloroquine because it detects very low concentrations (1 ng/ml);

21- *Anopheles* mosquitoes were not found inside the residential area of SNV;

22- Transmission of malaria infection in the control areas, is apparently both indoors and outdoors; suitable mosquitoes were found in both locations;

23- Transmission can occur throughout the entire year but is most likely after the rainy season when the density of *Anopheles* mosquitoes becomes higher;

24- *An. albitarsis*, if not the main vector for these areas, probably plays an important role in malaria transmission in all control areas due to its density in comparison to the other species found, its positivity for malaria sporozoites and its habit of feeding indoors in the residential areas;

25- *An. nuñeztovari* may also play an important role in

malaria transmission in CAB, where it was caught as immature and adult forms: it was found positive for *P. vivax, P.vivax*-variant VK247 (type 2) and *P. malariae;*

26- There are other species of *Anopheles* mosquitoes such as *An. oswaldoi* and *An. braziliensis* that also have a role in malaria transmission, since the first was found infected with *P.falciparum* and the second was the second most frequent species in ARR and was found positive for *P. malariae.*

27- *An. darlingi,* a very efective malaria vector was found in collections with human bait, in the vicinity of ARR and CAB, and not in SNV or adjacent forest;

28- The daily intake of chloroquinised salt in SNV has been implicated, at least partially, in the prevention of malaria infection in this area;

29- The lack of malaria infections in SNV although CS antibody studies indicate exposure to infected mosquitoes, suggests that there may be species in the area which do not readily transmit chloroquine-resistant *P. falciparum* strains. The absence of *An. darlingi* from SNV may perhaps be responsible for the apparent absence of chloroquine-resistant malaria there;

30- The programme for controlling malaria transmission in SNV, proved to be very effective.

7.1 Monitoring the presence and/or spread of resistance of falciparum and vivax malaria to the antimalarial drugs which have been or are used at present;

7.2 Trials in the application of new antimalarial drugs (halofantrine and artemisinin) recently introduced into Brazil and the evaluation of their efficacy and effects among these populations;

7.3 Studies of the diversity and polymorphism of the malaria parasites and/or candidate antigens for vaccines in order to assess the degree and distribution of such diversity and polymorphism;

7.4 Studies on the distribution of *P. vivax* variants to establish their clinical features, sensitivity to antimalarial drugs and the occurrence of relapses;

7.5 Nothing is known about innate susceptibility to malaria in Brazil. It would therefore be important to study the presence of the sickle cell gene, thalassaemias and G-6-PD deficiency in the Amazon region. An MHC study of association with malaria infectivity or severity may be of interest;

7.6 Investigation of the protective impact of chloroquinised salt within a selected population, carrying out a trial where its effect alone and together with other control measures could be evaluated. The population diet would also have to be surveyed in order to evaluate the real daily intake of this salt;

7.7 Studies to study the behaviour of the *Anopheles* species as vectors, and the capability of harbouring chloroquine sensitive and resistant *P. falciparum*;

7.8 Studies of the present levels of the sensitivity/ resistance of the *Anopheles* mosquitoes of these areas to insecticides, not only to DDT that has been used for many years, but also to others that

RECOMMENDATIONS

1 - The use of antimalarial drugs such as chloroquine and amodiaquine for treating falciparum malaria must be avoided in this study region, unless a rapid diagnostic test becomes available to determine if a patient carries a resistant or sensitive strain;

2 - Areas such as ARR and CAB, in which the residential area is too close to the forest, could try to establish a ring of protection by forest clearing or building houses about 200m away from the forest;

3 - For ARR, PT and CAB where breeding sites for the immature stages of *Anopheles* mosquitoes were found, a programme for their control could be established. Those breeding sites that are permanent, such as ponds, small rivers and the main river could be kept free of vegetation on their edges, free of the rubbish that is usually dropped into them, and be treated. Those that are temporary such as pools, creeks, flooded areas, could be removed and/or be kept clean;

3 - A sanitary education programme could be carried out in the control areas in order to provide them with basic, but important information about malaria transmission and to demonstrate how and why malaria transmission is prevented;

4 - The spraying of insecticides with residual effect could be continued with monitoring for the possible emergence of resistance of insecticides in the vectors;

6 - Transference of the knowledge and experience of the ICOMI programme for controlling malaria transmission to other similar companies settled in the Amazon region, would certainly contribute to the decrease of the morbidity of malaria in Brazil;

7 - At least, the following further studies to understand malaria in the areas where this study was carried out, would be valuable:

can be introduced into these areas in the future;

7.9 A study on the application of impregnated bednets or hammocks, the usually bed for people in the Amazon region and an evaluation of their efficacy;

Areas in this region of Amapá State would also be suitable, following these further studies, for coordinated intervention trials to establish a model for use in similar localities throughout the Amazon region.

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APPENDIX 1

PERSONAL DATA

ID number: . . . . . .

Name: . . . . . .

Sex:. . . . . **Age:**. . . . . **Place of birth**. . . . . .

Address: . . . . . .

How long have you been at the above address? . . . . . .

Past addresses (till 10 years ago):

1 - . . . . . period:. . . .

2 - . . . . . period:. . . .

3 - . . . . . period:. . . .

Do you use chloroquinized salt?

yes ( ); no ( ) If yes, for how long? . . . . . .

Do you use personal protection?

yes ( ); no ( ) If yes, which one? . . . . . .

Reported tropical diseases:

Malaria ( ) If yes, how many times? . . .

Leishmaniasis ( )

Filariasis ( )

Chagas' disease ( )

Toxoplasmosis ( )

Past malaria infection:

Type	**Date (year)**	**Probable area where infection acquired**
. . .	. . . . .	. . . . .
. . .	. . . . .	. . . . .
. . .	. . . . .	. . . . .

Are you using or have you used any medicine?

yes ( ); no ( ) If yes, which and for how long? . .

. . . . . .

Splenic rate: 0 ( ); 1 ( ); 2 ( ); 3 ( ); 4 ( )

NOTES: . . . . . .

. . . . . .

. . . . . .

APPENDIX 2

PAULINI'S METHOD

It is a spectrophotometric method for the dosage of chloroquine, that is performed as:

0.5 g of chloroquinised salt containing approximately 0.2% of chloroquine base, is dissolved in 100 ml of dH_2O. After vigorous agitation, if necessary, filtrate through dry paper. Then, the OD is determined in a spectrophotometer with 360 μ.

For obtaining the percentage of chloroquine base in the salt, the determined OD is multiplied by the factor 0.346.

APPENDIX 3

Glycerolyte 57 solution

It is a sterile nonpyrogenic solution:

Glycerine USP	100 ml
Sodium lactate	1.6 g
Potassium chloride	30.0 mg

It is buffered with:

Monobasic sodium phosphate	1.6 mg
Dibasic sodium phosphate	124.2 mg

pH approx. 6.8

FENWAL LABORATORIES
Division of Travenol Laboratories, Inc.
Deerfield, IL 600 15, USA
Distributed in Canada by
Travenol Canada Inc.
Mississauga, Ontario, Canada 14V 1J3.

APPENDIX 4

Blocking buffer used in ELISA test for the detection of malaria sporozoites in mosquitoes.

Casein (0.5%)	5.00 g
0.1N NaOH	100.00 ml
PBS, pH 7.4	900.00 ml
Thimerosal (0.1%)	1.00 ml
Red phenol (0.1%)	200.00 µl

1- Dissolve the casein in 0.1N NaOH solution by ebullition;
2 - Add the PBS to the solution of casein, but slowly;
3 - Adjust the pH to 7.4 b with HCl; and
4 - Add the timerosal and red phenol

APPENDIX 5

METHOD OF ANALYSIS BY CARPENTER (DRAPER *ET AL.*, 1972)

The risk of infection can be estimated in areaswhere malaria is endemic by assuming that the inhabitants are at fairly constant risk of infection.

Suppose τ is a probability of a person being infected in a short interval of time (a day), the probability of a person never having been infected in **n** days (being serologically negative at age **n** days) is:

$$\text{Prob (-ve)} = (1 - \tau)^n \sim e^{-n\tau}$$

If age is measured as usual in years and $u = 365\tau$, then

Prob (-ve at age A in years) = $_e$ - uA and the probability of a person livingin the area being infected in a year is given by R,

R = probability of being infected in 1 year

= infection R

= 1 - $_e$ -u

Infection rate estimation of an area can be calculater by producing the number of people tested, and the proportion serologically positive in a sequence of age groups (children under one year omitted, due to presence of congenital antibodies).

According to the model in the **i**th group age A_i

$$\text{Proportion +ve} = 1 - {}_e\text{-}uA_i$$

consequently,

$$\text{-log (1-proportion +ve)} = ua_i$$

That is, if the proportiom of +ve is plotted on a reverse log scale, the model predictsa linear relationship with age apart from random sampling errors.

APPENDIX 6

SEQUENCE OF PRIMERS FOR *P. vivax* AND *P. falciparum*

1 - *P. vivax*

AL 60 GTC GGA ATT CAT GAA GAA CTT CAT TCT C
AL 61 CAG CGG ATC CTT AAT TGA ATA ATG CTA GG
AL 114 ATC AAC CAG GAG CAA ATG
AL 116 GGT GAT AGA GCA GAT GGA
AL 54 CCA TGC AGT GTA ACC TGT GGA

2 - *P. falciparum*

AL 52 CAG GAA ACA GCT ATG AC
AL 53 GTA AAA CGA CGG CCA GT
AL 3 AAC ACA AGG GTT CTA AAT GAA TTA
AL 9 AAT AAA AAC AAT CAA GGT AAT
AL 164 CAT GGG GAG GAT TCA GTT G

ABBREVIATIONS USED ON THE APPENDIX OF RESULTS

S	Sex
A	Age
MH	Malaria history
SL	slide
SR	spleen rate
IF	IFAT for *P. falciparum*
IV	IFAT for *P. vivax*
EB	ELISA for blood stages
CSF	ELISA for sporozoite of *P. falciaprum*
CSV	ELISA for sporozoite of *P. vivax* - type 1
CSK	ELISA for sporozoite of *P. vivax* - type 2
CSN	ELISA for sporozoite of *P. vivax* - type 3
CSM	ELISA for sporozoite of *P. malariae/P.brasilianum*
CHS	Chloroquinized salt
PF	*P. falciparum*
PV	*P. vivax*
MX	Mixed infection (*P. falciparum* + *P. vivax*)
ND	Not done
N	Negative
P	Positive

APPENDIX 7

TARGET AREA: SERRA DO NAVIO

CODE	S	A	MH	SR	HG	HK	CQUR	CQSE
ICO-001	F	25	N	0	ND	P	ND	12.5
ICO-002	F	5	N	0	ND	P	ND	11
ICO-003	M	3	N	0	ND	N	ND	10.7
ICO-004	F	6	N	0	ND	N	ND	10.1
ICO-005	M	9	N	0	ND	N	600	4.6
ICO-006	F	5	N	0	1655	N	1200	6.4
ICO-007	M	37	N	0	ND	P	10000	6.4
ICO-008	F	11	N	0	ND	N	ND	9
ICO-009	F	11	N	0	ND	N	ND	11.5
ICO-010	F	33	N	0	ND	N	ND	9.9
ICO-011	F	26	N	0	ND	P	940	6.3
ICO-012	F	5	N	0	ND	P	1200	9
ICO-013	M	4	N	0	ND	P	990	3.2
ICO-014	M	3	N	0	ND	P	490	8.5
ICO-015	M	1	N	0	ND	N	990	5.2
ICO-016	F	34	N	0	ND	P	1900	6.9
ICO-017	M	47	Y2	0	ND	P	990	8.1
ICO-018	F	9	N	0	ND	P	440	8.5
ICO-019	F	33	N	0	ND	P	760	8.6
ICO-020	F	17	N	0	ND	P	520	2
ICO-021	F	35	N	0	ND	N	730	18
ICO-022	M	26	N	0	ND	P	ND	8.1
ICO-023	F	4	N	0	ND	N	990	10.8
ICO-024	M	11	N	0	ND	N	1000	8
ICO-025	F	12	N	0	ND	N	560	8.5
ICO-026	F	2	N	0	ND	N	1000	8.6
ICO-027	F	30	N	0	ND	P	1700	6.5
ICO-028	F	18	N	0	ND	P	1600	11.5
ICO-029	F	14	N	0	ND	P	900	60
ICO-030	F	49	Y1	0	ND	N	450	8.5
ICO-031	F	46	N	0	ND	N	760	1
ICO-032	F	11	N	0	ND	N	950	10
ICO-033	M	14	N	0	ND	N	880	17
ICO-034	M	46	N	0	ND	N	700	11.5
ICO-035	M	7	N	0	ND	P	1200	2.6
ICO-036	F	8	N	0	>2500	N	860	6
ICO-037	F	29	N	0	ND	P	990	6.6
ICO-038	M	10	N	0	ND	N	730	3.8
ICO-039	M	26	N	O	ND	P	950	3.2
ICO-040	M	38	Y1	0	ND	P	700	1.5
ICO-041	M	37	Y2	0	ND	P	990	3.4
ICO-042	M	8	N	0	ND	N	360	1.6
ICO-043	M	11	N	0	1280	N	250	2.9
ICO-044	F	9	N	0	ND	N	240	3.5
ICO-045	F	9	N	0	ND	P	330	5.1
ICO-046	F	31	N	0	ND	N	220	2.3
ICO-047	M	29	N	0	ND	P	290	4.8
ICO-048	F	2	N	0	ND	N	220	10
ICO-049	F	1	N	0	ND	N	310	7.5
ICO-050	F	27	N	0	ND	P	1600	8.5

ICO-051	F	8	N	0	ND	P	320	6.2
ICO-052	F	46	N	0	ND	N	700	1
ICO-053	M	57	N	0	ND	P	340	2.5
ICO-054	M	18	N	0	ND	N	720	1
ICO-055	F	37	N	0	ND	N	310	2.4
ICO-056	M	14	N	0	ND	P	800	1
ICO-057	F	13	N	0	ND	P	ND	1.6
ICO-058	M	10	N	0	ND	P	700	8
ICO-059	M	36	N	0	ND	N	420	1
ICO-060	F	8	N	0	ND	N	210	4.9
ICO-061	F	5	N	0	ND	N	360	2.7
ICO-062	F	3	N	0	ND	N	990	1
ICO-063	M	7	N	0	ND	N	410	6.1
ICO-064	F	25	N	0	ND	P	780	1.7
ICO-065	M	31	N	0	ND	P	250	5.1
ICO-066	F	36	Y1	0	ND	N	8500	6.6
ICO-067	M	14	N	0	ND	N	3911	13
ICO-068	M	12	N	0	ND	N	1050	10
ICO-069	M	40	Y1	0	ND	N	370	6.7
ICO-070	M	32	N	0	ND	N	1900	2
ICO-071	F	33	N	0	ND	P	4000	12
ICO-072	F	5	N	0	ND	N	480	19
ICO-073	F	2	N	0	250	N	410	10
ICO-074	F	36	Y1	0	ND	P	210	2.3
ICO-075	M	11	N	0	ND	N	753	4
ICO-076	M	14	N	0	ND	N	110	2.6
ICO-077	M	12	N	0	ND	N	160	ND
ICO-078	M	50	Y1	0	ND	P	184	5.6
ICO-079	M	6	N	0	ND	P	180	1.8
ICO-080	F	17	N	0	ND	P	150	1
ICO-081	M	14	N	0	ND	P	220	4.4
ICO-082	F	40	Y1	0	ND	N	220	2.5
ICO-083	M	39	N	0	ND	P	2349	4.9
ICO-084	M	38	N	0	ND	N	160	1
ICO-085	M	45	Y1	0	ND	P	230	2.4
ICO-086	M	37	Y4	0	ND	P	190	1
ICO-087	M	23	Y1	0	ND	P	200	6.4
ICO-088	F	25	N	0	ND	N	260	8.2
ICO-089	F	6	N	0	ND	P	220	8.5
ICO-090	M	1	N	0	ND	N	190	12
ICO-091	M	21	N	0	ND	P	230	9
ICO-092	M	16	N	0	ND	P	280	2.1
ICO-093	F	33	N	0	ND	P	260	1.9
ICO-094	F	5	N	0	ND	P	180	2.6
ICO-095	F	4	N	0	ND	P	210	5.1
ICO-096	M	34	N	0	ND	P	290	1.2
ICO-097	M	8	N	0	ND	P	310	1.3
ICO-098	M	24	Y4	0	ND	N	360	1.5
ICO-099	F	22	N	0	ND	P	410	1.3
ICO-100	F	3	N	0	ND	N	230	1.2
ICO-101	F	11	N	0	ND	P	250	1.1
ICO-102	F	5	N	0	ND	N	200	1.1
ICO-103	F	11	N	0	ND	N	220	1.2
ICO-104	F	36	Y1	0	ND	P	1200	1.2
ICO-105	M	15	Y1	0	ND	P	420	1.2
ICO-106	M	38	N	0	ND	P	300	1.3

ICO-107	M	31	N	0	ND	P	260	1.3
ICO-108	F	30	Y1	0	ND	N	212	0
ICO-109	F	5	N	0	ND	N	150	1.2
ICO-110	F	13	N	0	ND	N	155	1.3
ICO-111	M	3	N	0	1160	N	240	1.2
ICO-112	F	7	N	0	ND	N	ND	1.2
ICO-113	F	10	N	0	1100	P	155	0
ICO-114	F	31	N	0	ND	N	ND	1.2
ICO-115	M	42	N	0	ND	N	ND	1.2
ICO-116	M	31	N	0	ND	N	155	1.2
ICO-117	F	28	N	0	ND	N	230	1.1
ICO-118	M	9	N	0	ND	N	260	1.2
ICO-119	M	7	N	0	1200	N	180	1.1
ICO-120	F	4	N	0	480	ND	ND	0
ICO-121	M	2	N	0	ND	ND	540	1.3
ICO-122	F	19	N	0	ND	P	210	1.3
ICO-123	M	23	N	0	ND	P	170	0
ICO-124	F	3	N	0	2560	N	155	0
ICO-125	F	25	Y2	0	ND	N	155	0
ICO-126	M	24	N	0	ND	N	155	0
ICO-127	F	8	N	0	ND	P	180	1.1
ICO-128	M	7	N	0	ND	N	155	0
ICO-129	F	6	N	0	ND	P	ND	0
ICO-130	F	33	N	0	ND	N	130	0
ICO-131	M	30	Y3	0	ND	N	330	1.1
ICO-132	M	48	Y4	0	ND	N	220	2.1
ICO-133	F	42	Y1	0	ND	P	275	1
ICO-134	F	3	N	0	ND	P	260	2.7
ICO-135	F	22	N	0	ND	N	210	1
ICO-136	M	13	Y1	0	>2500	N	180	2.4
ICO-137	M	20	N	0	ND	N	ND	1
ICO-138	F	21	N	0	ND	N	270	1.6
ICO-139	M	51	Y1	0	ND	P	850	7.5
ICO-140	F	46	Y1	0	ND	N	160	1
ICO-141	M	6	N	0	1490	N	ND	4.4
ICO-171	M	43	N	0	ND	P	180	5
ICO-172	F	36	N	0	ND	P	150	3.5
ICO-173	M	12	N	0	ND	P	180	6.5
ICO-174	M	10	N	0	ND	P	5195	6.5
ICO-175	M	3	N	0	ND	P	4268	5.1
ICO-176	M	29	N	0	ND	N	150	1.8
ICO-177	F	24	N	0	ND	N	150	2.9
ICO-178	F	13	N	0	ND	N	ND	ND
ICO-202	M	5	N	0	<250	N	1750	6.8
ICO-203	F	31	N	0	ND	N	2100	8.2
ICO-204	M	35	Y1	0	ND	P	1460	7.5
ICO-205	F	43	N	0	ND	N	1300	6
ICO-206	M	53	Y1	0	ND	P	6200	4.2
ICO-207	F	3	N	0	1270	P	2100	1
ICO-208	F	10	N	0	ND	N	6200	10
ICO-209	M	26	N	0	ND	N	1450	8.5
ICO-210	M	26	N	0	ND	N	1100	2.8
ICO-211	M	35	N	0	ND	P	1400	1.6
ICO-212	F	26	N	0	ND	N	2100	1.55
ICO-213	F	11	N	0	ND	N	1500	4.2
ICO-214	F	3	N	0	ND	P	1055	1.3

ICO-215	M	8	N	0	ND	P	5800	1.3
ICO-216	F	5	N	0	1465	P	ND	1.4
ICO-217	F	29	N	0	ND	P	1300	1.3
ICO-218	M	33	Y2	0	ND	P	6000	1.55
ICO-219	F	14	N	0	ND	P	980	1.4
ICO-220	F	7	N	0	ND	P	1070	1.3
ICO-221	F	5	N	0	ND	N	1080	1.3
ICO-222	F	10	N	0	ND	P	5200	1.5
ICO-223	F	12	N	0	ND	P	870	1.55
ICO-224	F	39	N	0	ND	P	1850	1.3
ICO-225	M	41	N	0	ND	N	8600	1.3
ICO-245	M	33	N	0	ND	N	1100	1.3
ICO-246	M	37	N	0	ND	P	2245	5
ICO-247	F	31	N	0	ND	P	2348	13
ICO-248	M	9	N	0	ND	N	1148	10
ICO-249	F	6	N	0	ND	N	880	5
ICO-250	F	4	N	0	ND	P	ND	6
SNV-001	M	33	N	0	ND	P	990	2.3
SNV-002	F	25	N	0	ND	ND	ND	3.6
SNV-003	F	2	N	0	ND	ND	ND	0
SNV-004	F	32	N	0	ND	P	550	2.9
SNV-005	M	3	N	0	ND	ND	ND	1.1
SNV-006	F	16	N	0	ND	P	280	2.7
SNV-007	M	58	Y1	0	ND	P	1050	6.1
SNV-008	F	26	N	0	ND	ND	ND	3.0
SNV-009	M	7	N	0	1200	P	580	4.3
SNV-010	F	45	N	0	ND	P	1200	5.8
SNV-011	F	24	N	0	ND	P	970	ND
SNV-012	F	13	N	0	1430	N	350	ND
SNV-013	F	59	Y4	0	ND	P	1180	ND
SNV-014	F	24	N	0	ND	ND	ND	ND
SNV-015	F	2	N	0	1020	N	130	ND
SNV-016	F	25	Y1	0	ND	P	2100	ND
SNV-017	M	4	N	0	2030	N	150	ND
SNV-018	F	2	N	0	ND	N	ND	ND
SNV-019	F	29	N	0	ND	ND	ND	ND
SNV-020	F	3	N	0	1900	N	ND	ND
SNV-021	F	27	N	0	ND	P	ND	ND
SNV-022	M	3	N	0	ND	P	880	ND
SNV-023	F	31	N	0	ND	P	630	ND
SNV-024	F	3	N	0	ND	N	ND	ND
SNV-025	F	12	N	0	ND	ND	ND	ND
SNV-026	F	32	N	0	ND	ND	ND	ND
SNV-027	F	50	N	0	ND	ND	ND	ND
SNV-028	F	28	N	0	ND	P	1250	ND
SNV-029	F	4	N	0	ND	N	145	1.1
SNV-030	F	15	N	0	ND	N	130	1.2
SNV-031	F	25	N	0	ND	P	775	ND
SNV-032	F	25	N	0	ND	P	710	ND
SNV-033	F	12	N	0	ND	P	1450	1.7
SNV-037	F	29	N	0	ND	P	2100	18
SNV-041	F	20	N	0	ND	P	1030	21
SNV-058	F	41	N	0	ND	P	950	1.3
SNV-060	M	48	N	0	ND	P	780	1.2
SNV-061	M	36	N	0	ND	P	810	3.1
SNV-062	F	33	N	0	ND	P	420	1.5

SNV-063	F	14	N	0	ND	P	390	2.7
SNV-064	F	12	N	0	ND	ND	ND	ND
SNV-065	M	9	N	0	ND	ND	ND	2.1
SNV-066	M	11	N	0	ND	P	150	1.1
SNV-067	M	7	N	0	ND	ND	ND	0
SNV-068	F	37	N	0	ND	P	2310	10
SNV-069	F	10	N	0	ND	P	1740	2.6
SNV-070	F	4	N	0	ND	N	290	1.3
SNV-071	F	32	Y1	0	ND	P	230	1.5
SNV-072	M	29	N	0	ND	N	120	1.1
SNV-073	F	22	N	0	ND	N	0	0
SNV-074	F	20	N	0	ND	P	370	1.2
SNV-075	F	28	N	0	ND	P	280	2.3
SNV-076	M	44	Y1	0	ND	P	1300	3.0
SNV-077	F	39	N	0	ND	P	870	1.8
SNV-078	M	48	N	0	ND	ND	ND	1.7
SNV-079	F	31	N	0	ND	P	530	1.6
SNV-080	F	19	N	0	ND	P	490	1.3
SNV-081	F	28	N	0	ND	ND	ND	ND
SNV-082	F	51	Y1	0	ND	P	270	0
SNV-083	F	22	N	0	ND	P	315	1.1
SNV-084	F	43	N	0	ND	P	80	2.0
SNV-085	F	36	N	0	ND	ND	ND	2.1
SNV-086	F	4	N	0	1660	ND	ND	0
SNV-087	F	36	N	0	ND	P	ND	2.2
SNV-088	F	29	N	0	ND	P	1540	5.2
SNV-089	M	4	N	0	ND	N	320	1.3
SNV-090	F	16	N	0	ND	P	1190	2.5
SNV-091	F	24	N	0	ND	P	740	1.9
SNV-092	F	36	N	0	ND	ND	ND	1.5
SNV-093	F	12	N	0	ND	ND	ND	1.5
SNV-094	F	31	N	0	ND	ND	ND	ND
SNV-095	F	21	N	0	ND	ND	ND	ND
SNV-099	F	13	N	0	ND	ND	ND	2.3
SNV-100	F	63	N	0	ND	ND	ND	3.5
SNV-101	F	15	N	0	ND	ND	ND	4.9
SNV-102	F	28	N	0	ND	ND	ND	11
SNV-103	F	15	N	0	ND	P	975	2.4
SNV-104	F	46	N	0	ND	P	850	1.3
SNV-105	F	13	N	0	ND	P	1070	3.5
SNV-106	F	20	Y1	0	ND	P	1350	3.3
SNV-107	F	34	N	0	ND	P	2160	9.8

TARGET AREA: SERRA DO NAVIO - 1st sample

CODE	SL	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
ICO-001	N	N	N	20	N	N	N	N	N
ICO-002	N	N	N	20	N	N	N	N	N
ICO-003	N	N	N	20	N	N	N	N	N
ICO-004	N	N	N	N	N	N	N	N	N
ICO-005	N	N	N	20	N	N	N	N	N
ICO-006	N	N	N	20	N	N	N	N	N
ICO-007	N	N	80	N	N	N	P	N	N
ICO-008	N	N	N	N	N	N	P	N	N
ICO-009	N	N	N	20	N	N	P	N	N
ICO-010	N	N	N	N	N	N	N	N	N
ICO-011	N	N	N	N	N	N	N	N	N
ICO-012	N	N	20	N	N	N	N	N	N
ICO-013	N	N	N	N	N	N	N	N	N
ICO-014	N	N	N	N	N	N	N	N	N
ICO-015	N	N	N	N	N	N	N	N	N
ICO-016	N	N	N	N	N	N	N	N	N
ICO-017	N	N	N	N	N	N	N	N	N
ICO-018	N	N	N	N	N	N	N	N	N
ICO-019	N	N	N	N	N	N	P	N	N
ICO-020	N	N	N	N	N	N	P	N	N
ICO-021	N	80	N	N	N	N	N	P	N
ICO-022	N	N	N	N	N	P	P	N	N
ICO-023	N	N	N	N	N	N	N	N	N
ICO-024	N	N	N	N	N	N	N	N	N
ICO-025	N	N	N	20	N	N	N	N	N
ICO-026	N	20	N	N	N	N	N	N	N
ICO-027	N	20	N	N	N	N	N	N	N
ICO-028	N	N	N	N	N	N	N	N	N
ICO-029	N	N	40	N	N	N	N	N	N
ICO-030	N	20	N	20	N	N	N	N	N
ICO-031	N	20	N	N	N	N	N	N	N
ICO-032	N	N	N	N	N	N	N	N	N
ICO-033	N	N	N	N	N	N	P	N	N
ICO-034	N	N	N	N	N	N	N	N	N
ICO-035	N	N	N	N	N	N	N	N	N
ICO-036	N	N	N	N	N	N	N	N	N
ICO-037	N	N	N	N	N	N	N	N	N
ICO-038	N	N	N	N	N	N	P	N	N
ICO-039	N	N	N	N	P	N	N	N	N
ICO-040	N	20	N	N	P	N	N	N	N
ICO-041	N	40	N	N	N	N	N	N	N
ICO-042	N	N	N	N	N	N	P	P	N
ICO-043	N	N	N	N	N	N	N	P	P
ICO-044	N	N	N	N	P	P	N	N	N
ICO-045	N	N	N	N	N	N	N	N	N
ICO-046	N	N	40	N	N	N	P	N	N
ICO-047	N	80	N	N	N	N	N	N	N
ICO-048	N	N	N	N	N	N	N	N	N
ICO-049	N	N	N	N	N	N	N	N	N
ICO-050	N	N	40	N	N	N	N	N	P

ICO-051	N	N	N	N	N	N	N	N	P
ICO-052	N	40	N	N	N	N	N	N	N
ICO-053	N	20	N	N	N	N	N	P	N
ICO-054	N	N	N	N	N	N	N	N	N
ICO-055	N	20	N	N	N	N	N	N	N
ICO-056	N	N	N	N	N	N	N	N	P
ICO-057	N	N	N	N	N	N	N	N	N
ICO-058	N	N	N	N	N	N	N	N	N
ICO-059	N	20	N	N	N	P	N	N	N
ICO-060	N	N	N	N	P	N	N	P	P
ICO-061	N	N	N	N	P	N	N	N	N
ICO-062	N	N	N	160	P	N	N	N	N
ICO-063	N	N	N	N	N	N	N	N	N
ICO-064	N	N	N	N	N	N	N	N	P
ICO-065	N	20	N	N	N	N	N	N	N
ICO-066	N	N	N	N	N	N	N	P	N
ICO-067	N	N	N	N	N	N	N	N	N
ICO-068	N	N	N	N	N	N	N	N	N
ICO-069	N	N	N	N	N	N	N	N	N
ICO-070	N	N	N	N	N	N	N	N	N
ICO-071	N	N	N	N	N	N	P	P	P
ICO-072	N	N	N	20	N	N	N	N	N
ICO-073	N	N	N	N	N	N	N	N	N
ICO-074	N	20	N	N	N	N	P	N	N
ICO-075	N	N	N	N	N	P	N	N	N
ICO-076	N	N	N	N	N	P	P	P	N
ICO-077	N	N	N	N	N	N	N	N	N
ICO-078	N	80	N	20	N	P	N	P	N
ICO-079	N	N	N	N	N	N	N	N	N
ICO-080	N	N	N	N	N	P	P	N	P
ICO-081	N	N	N	N	N	N	N	N	P
ICO-082	N	N	N	N	N	N	N	N	N
ICO-083	N	N	N	N	N	N	N	N	N
ICO-084	N	N	N	N	N	N	N	N	N
ICO-085	N	N	N	N	N	N	N	N	N
ICO-086	N	N	N	N	N	N	N	N	N
ICO-087	N	N	N	N	N	P	N	N	N
ICO-088	N	N	N	N	N	N	N	N	N
ICO-089	N	N	N	N	N	N	N	N	N
ICO-090	N	N	N	N	N	N	N	N	N
ICO-091	N	N	N	N	N	N	N	N	N
ICO-092	N	N	N	N	N	N	N	N	N
ICO-093	N	N	N	N	N	N	N	N	N
ICO-094	N	N	N	N	N	N	N	N	N
ICO-095	N	N	N	N	N	N	N	N	N
ICO-096	N	N	N	N	N	N	N	N	N
ICO-097	N	N	N	N	N	N	N	N	N
ICO-098	N	N	N	N	N	N	N	N	N
ICO-099	N	N	N	N	N	N	N	N	N
ICO-100	N	N	N	N	N	N	N	N	N
ICO-101	N	N	N	N	N	N	N	N	P
ICO-102	N	N	N	N	P	P	N	N	N
ICO-103	N	N	N	N	N	N	N	N	N
ICO-104	N	N	N	N	N	N	N	N	N
ICO-105	N	N	N	N	P	N	N	N	N
ICO-106	N	N	N	N	P	N	N	N	N

ICO-107	N	N	N	N	N	N	N	N	N
ICO-108	N	N	N	N	P	N	N	N	N
ICO-109	N	N	N	N	N	N	N	N	N
ICO-110	N	N	N	N	N	N	N	N	N
ICO-111	N	N	N	N	N	N	N	N	N
ICO-112	N	N	N	N	N	N	N	N	N
ICO-113	N	N	N	20	N	N	N	N	N
ICO-114	N	N	N	20	N	N	N	N	N
ICO-115	N	N	N	N	P	P	P	P	P
ICO-116	N	N	N	N	P	N	N	N	N
ICO-117	N	N	N	N	P	N	N	N	N
ICO-118	N	N	N	N	N	N	N	N	N
ICO-119	N	N	N	N	N	N	N	N	N
ICO-120	N	N	N	20	N	N	N	N	N
ICO-121	N	N	N	N	N	N	N	N	N
ICO-122	N	20	N	N	P	N	N	N	N
ICO-123	N	N	N	N	N	N	N	N	N
ICO-124	N	N	N	N	N	N	N	N	N
ICO-125	N	N	N	20	N	N	N	N	N
ICO-126	N	N	N	N	N	N	N	N	N
ICO-127	N	N	N	N	N	N	N	N	N
ICO-128	N	N	N	N	N	N	N	N	N
ICO-129	N	N	N	N	N	N	N	N	N
ICO-130	N	N	N	N	N	N	N	N	N
ICO-131	N	N	N	20	N	N	N	N	N
ICO-132	N	640	N	N	N	P	N	N	N
ICO-133	N	N	N	N	N	N	N	N	N
ICO-134	N	N	N	N	N	N	N	N	N
ICO-135	N	N	N	N	N	N	N	N	P
ICO-136	N	N	N	20	P	N	N	N	N
ICO-137	N	N	N	N	N	N	N	N	N
ICO-138	N	N	N	N	N	N	N	P	N
ICO-139	N	80	N	40	P	N	N	N	N
ICO-140	N	N	N	N	N	N	N	N	N
ICO-141	N	N	N	20	N	N	N	N	N
ICO-171	N	N	N	N	N	N	N	N	N
ICO-172	N	N	N	N	N	N	N	N	N
ICO-173	N	N	N	20	N	N	N	N	N
ICO-174	N	N	N	40	N	P	N	N	N
ICO-175	N	N	N	20	N	N	N	N	N
ICO-176	N	N	N	20	N	N	N	N	N
ICO-177	N	N	N	20	N	N	N	N	P
ICO-178	N	N	N	N	N	N	N	N	N
ICO-202	N	N	N	N	P	P	N	P	N
ICO-203	N	N	N	N	N	N	N	N	N
ICO-204	N	N	N	N	N	N	N	N	N
ICO-205	N	N	N	N	N	P	N	P	N
ICO-206	N	N	N	N	N	N	N	P	N
ICO-207	N	N	N	N	N	N	N	N	N
ICO-208	N	N	N	N	N	N	N	N	N
ICO-209	N	N	N	20	N	P	N	N	N
ICO-210	N	N	N	20	N	P	N	N	N
ICO-211	N	N	N	N	P	N	N	N	P
ICO-212	N	N	N	20	P	N	N	N	N
ICO-213	N	N	N	20	N	N	N	N	N
ICO-214	N	N	N	N	N	N	N	N	N

ICO-215	N	N	N	20	N	P	N	N	N
ICO-216	N	N	N	20	N	N	N	N	N
ICO-217	N	N	N	N	P	P	N	P	P
ICO-218	N	N	N	N	P	P	N	P	P
ICO-219	N	N	N	N	N	P	N	N	N
ICO-220	N	N	N	N	N	N	N	N	N
ICO-221	N	N	N	20	N	N	N	N	P
ICO-222	N	N	N	20	N	N	N	N	P
ICO-223	N	N	N	40	N	N	N	N	P
ICO-224	N	N	N	40	P	N	N	N	P
ICO-225	N	N	N	N	N	P	N	N	P
ICO-245	N	40	N	20	N	N	N	N	P
ICO-246	N	N	N	20	P	P	N	N	P
ICO-247	N	N	N	20	N	P	N	N	P
ICO-248	N	N	N	40	N	N	N	N	N
ICO-249	N	N	N	40	N	N	N	N	P
ICO-250	N	N	N	N	N	N	N	N	P
SNV-001	N	N	N	N	N	N	N	N	N
SNV-002	N	N	N	N	N	N	N	N	N
SNV-003	N	N	N	N	N	N	N	N	N
SNV-004	N	N	N	N	N	N	N	N	N
SNV-005	N	N	N	N	N	N	N	N	N
SNV-006	N	N	N	N	N	N	N	N	N
SNV-007	N	N	N	N	P	N	N	N	N
SNV-008	N	N	N	N	N	N	N	N	N
SNV-009	N	N	N	N	N	N	N	N	N
SNV-010	N	N	N	N	N	N	N	N	N
SNV-011	N	N	N	N	P	N	N	N	N
SNV-012	N	N	N	N	N	N	N	N	N
SNV-013	N	N	N	20	N	N	N	N	N
SNV-014	N	N	N	N	N	N	N	N	N
SNV-015	N	N	N	N	N	N	N	N	N
SNV-016	N	N	N	N	N	N	N	N	N
SNV-017	N	N	N	N	N	N	N	N	N
SNV-018	N	N	N	N	N	N	N	N	N
SNV-019	N	N	N	N	N	N	N	N	N
SNV-020	N	N	N	N	N	N	N	N	N
SNV-021	N	N	N	N	N	N	N	N	N
SNV-022	N	N	N	20	N	N	N	N	N
SNV-023	N	N	N	N	N	N	N	N	N
SNV-024	N	N	N	N	N	N	N	N	N
SNV-025	N	N	N	N	N	N	N	N	N
SNV-026	N	N	N	N	N	N	N	N	N
SNV-027	N	N	N	N	N	N	N	N	N
SNV-028	N	N	N	N	N	N	N	N	N
SNV-029	N	N	N	N	N	N	N	N	N
SNV-030	N	N	N	N	N	N	N	N	N
SNV-031	N	N	N	N	N	N	N	N	N
SNV-032	N	N	N	N	N	N	N	N	N
SNV-033	N	N	N	N	N	N	N	N	N
SNV-037	N	N	N	N	N	N	N	N	N
SNV-041	N	N	N	N	P	N	N	N	N
SNV-058	N	N	N	N	P	N	N	N	N
SNV-060	N	N	N	N	N	N	N	N	N
SNV-061	N	N	N	N	N	N	N	N	N
SNV-062	N	N	N	N	N	N	N	N	N

SNV-063	N	N	N	N	N	N	N	N	N
SNV-064	N	N	N	N	N	N	N	N	N
SNV-065	N	N	N	N	N	N	N	N	N
SNV-066	N	N	N	N	N	N	N	N	N
SNV-067	N	N	N	N	N	N	N	N	N
SNV-068	N	N	N	N	N	N	N	N	N
SNV-069	N	N	N	N	N	N	N	N	N
SNV-070	N	N	N	N	N	N	N	N	N
SNV-071	N	N	N	N	P	N	N	N	N
SNV-072	N	N	N	N	N	N	N	N	N
SNV-073	N	N	N	20	N	N	N	N	N
SNV-074	N	N	N	N	N	N	N	N	N
SNV-075	N	N	N	N	N	N	N	N	N
SNV-076	N	N	N	N	N	N	N	N	N
SNV-077	N	N	N	N	N	N	N	N	N
SNV-078	N	N	N	N	N	N	N	N	N
SNV-079	N	N	N	N	N	N	N	N	N
SNV-080	N	N	N	N	N	N	N	N	N
SNV-081	N	N	N	N	N	N	N	N	N
SNV-082	N	N	N	N	N	N	N	N	N
SNV-083	N	N	N	N	N	N	N	N	N
SNV-084	N	N	N	N	N	N	N	N	N
SNV-085	N	N	N	N	N	N	N	N	N
SNV-086	N	N	N	N	N	N	N	N	N
SNV-087	N	N	N	N	N	N	N	N	N
SNV-088	N	N	N	N	N	N	N	N	N
SNV-089	N	N	N	N	N	N	N	N	N
SNV-090	N	N	N	N	N	N	N	N	N
SNV-091	N	N	N	N	N	N	N	N	N
SNV-092	N	N	N	N	N	N	N	N	N
SNV-093	N	N	N	N	N	N	N	N	N
SNV-094	N	N	N	N	N	N	N	N	N
SNV-095	N	N	N	N	N	N	N	N	N
SNV-099	N	N	N	20	N	N	N	N	N
SNV-100	N	N	N	N	N	N	N	N	N
SNV-101	N	N	N	N	N	N	N	N	N
SNV-102	N	N	N	N	N	N	N	N	N
SNV-103	N	N	N	N	N	N	N	N	N
SNV-104	N	N	N	N	N	N	N	N	N
SNV-105	N	N	N	N	N	N	N	N	N
SNV-106	N	N	N	N	N	N	N	N	N
SNV-107	N	N	N	N	N	N	N	N	N

TARGET AREA: SERRA DO NAVIO - 2nd sample

CODE	SL	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
ICO-001	N	N	N	40	N	N	N	N	N
ICO-002	N	N	N	20	N	N	N	N	N
ICO-003	N	N	N	20	N	N	N	N	N
ICO-004	N	N	N	N	N	N	N	N	N
ICO-005	N	N	N	20	N	N	N	N	N
ICO-006	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-007	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-008	N	N	N	N	N	N	N	N	N
ICO-009	N	N	N	N	N	N	N	N	N
ICO-010	N	N	N	N	N	N	N	N	N
ICO-011	N	N	N	N	N	N	P	P	N
ICO-012	N	N	N	N	N	N	N	N	N
ICO-013	N	N	N	N	N	N	N	N	N
ICO-014	N	N	N	20	N	N	N	N	N
ICO-015	N	N	N	N	N	N	N	N	N
ICO-016	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-017	N	N	N	N	N	N	N	N	N
ICO-018	N	N	N	N	N	N	N	N	N
ICO-019	N	N	N	N	N	N	P	N	N
ICO-020	N	N	N	N	P	N	P	N	N
ICO-021	N	20	N	N	N	N	P	N	P
ICO-022	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-023	N	N	N	N	N	N	N	N	N
ICO-024	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-025	N	N	N	N	N	N	N	N	N
ICO-026	N	N	N	N	N	N	N	N	N
ICO-027	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-028	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-029	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-030	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-031	N	20	N	N	N	N	N	N	N
ICO-032	N	N	N	N	N	N	N	N	N
ICO-033	N	N	N	N	N	N	N	N	N
ICO-034	N	N	N	N	N	N	N	N	N
ICO-035	N	N	N	20	N	N	N	N	N
ICO-036	N	N	20	N	N	P	N	N	N
ICO-037	N	N	N	N	N	N	N	N	N
ICO-038	N	N	N	N	N	N	P	N	N
ICO-039	N	N	N	N	P	N	N	N	N
ICO-040	N	20	N	N	N	N	N	N	N
ICO-041	N	20	N	N	N	N	N	N	N
ICO-042	N	N	N	N	N	N	P	P	N
ICO-043	N	N	N	N	P	N	N	N	P
ICO-044	N	N	N	N	N	N	N	N	N
ICO-045	N	N	N	N	P	N	P	N	N
ICO-046	N	N	40	N	N	N	N	N	N
ICO-047	N	80	N	N	N	N	N	P	N
ICO-048	N	N	N	N	N	N	N	N	N
ICO-049	N	N	N	N	N	N	N	N	N
ICO-050	N	N	80	N	N	N	N	N	N
ICO-051	N	N	N	N	N	N	N	N	N

ICO-052	N	40	N	N	N	N	N	N	N
ICO-053	N	20	N	N	N	N	N	N	N
ICO-054	N	N	N	N	N	P	N	N	N
ICO-055	N	20	N	N	N	N	N	N	N
ICO-056	N	N	N	N	N	N	N	P	P
ICO-057	N	N	N	N	N	N	N	N	N
ICO-058	N	N	N	N	N	N	N	N	N
ICO-059	N	20	N	N	N	P	N	N	N
ICO-060	N	N	N	N	N	N	N	N	N
ICO-061	N	N	N	N	N	N	N	N	N
ICO-062	N	N	N	160	N	N	N	N	N
ICO-063	N	N	N	N	N	N	N	N	N
ICO-064	N	N	N	N	N	N	N	N	N
ICO-065	N	40	N	N	N	N	N	P	P
ICO-066	N	N	N	N	N	N	N	N	N
ICO-067	N	N	N	N	N	N	N	N	N
ICO-068	N	N	N	N	N	N	N	N	N
ICO-069	N	N	N	N	N	N	N	N	N
ICO-070	N	N	N	N	N	N	P	N	N
ICO-071	N	N	N	N	N	N	P	P	P
ICO-072	N	N	N	20	N	P	P	P	N
ICO-073	N	N	N	N	N	N	N	N	N
ICO-074	N	N	N	N	N	N	N	N	N
ICO-075	N	N	N	N	N	P	N	N	N
ICO-076	N	N	N	N	N	P	N	P	N
ICO-077	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-078	N	40	N	20	N	N	N	N	N
ICO-079	N	N	N	N	N	N	N	N	N
ICO-080	N	N	N	N	N	N	N	N	N
ICO-081	N	N	N	N	N	N	N	N	N
ICO-082	N	N	N	N	N	P	P	P	P
ICO-083	N	N	N	N	N	N	N	N	N
ICO-084	N	N	N	N	N	N	N	N	N
ICO-085	N	N	N	N	N	N	N	N	N
ICO-086	N	N	N	N	N	N	N	N	N
ICO-087	N	N	N	N	N	N	N	N	N
ICO-088	N	N	N	N	N	N	N	N	N
ICO-089	N	N	N	N	N	N	N	N	N
ICO-090	N	N	N	N	N	N	N	N	N
ICO-091	N	N	N	N	N	N	N	N	N
ICO-092	N	N	N	N	N	N	N	N	N
ICO-093	N	N	N	N	N	N	P	N	N
ICO-094	N	N	N	N	N	N	N	N	N
ICO-095	N	N	N	N	N	N	N	N	N
ICO-096	N	N	N	N	N	N	N	N	N
ICO-097	N	N	N	N	N	N	N	N	N
ICO-098	N	N	N	N	N	N	N	N	N
ICO-099	N	N	N	N	N	N	N	N	N
ICO-100	N	N	N	N	N	N	N	N	N
ICO-101	N	N	N	N	N	N	P	N	P
ICO-102	N	N	N	N	N	N	N	N	N
ICO-103	N	N	N	N	N	N	N	N	N
ICO-104	N	N	N	N	P	N	N	N	N
ICO-105	N	N	N	N	N	N	N	N	N
ICO-106	N	N	N	N	P	N	N	N	N
ICO-107	ND	ND	ND	ND	ND	ND	ND	ND	ND

ICO-108	N	N	N	N	N	N	N	N	N
ICO-109	N	N	N	N	N	N	N	N	N
ICO-110	N	N	N	N	N	N	N	N	N
ICO-111	N	N	N	N	N	N	N	N	N
ICO-112	N	N	N	20	N	N	N	N	N
ICO-113	N	N	N	20	N	N	N	N	N
ICO-114	N	N	N	20	N	N	N	N	N
ICO-115	N	N	N	N	P	N	N	N	P
ICO-116	N	N	N	N	N	N	N	N	N
ICO-117	N	N	N	N	N	N	N	N	N
ICO-118	N	N	N	N	N	N	N	N	N
ICO-119	N	N	N	20	N	N	N	N	N
ICO-120	N	N	N	20	N	N	N	N	N
ICO-121	N	N	N	40	N	N	N	N	N
ICO-122	N	N	N	N	P	N	N	N	N
ICO-123	N	N	N	N	P	P	P	P	P
ICO-124	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-125	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-126	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-127	N	N	N	N	P	N	N	N	N
ICO-128	N	N	N	20	N	N	N	N	N
ICO-129	N	N	N	N	N	N	N	N	N
ICO-130	N	N	N	N	N	N	N	N	N
ICO-131	N	N	N	N	N	N	N	N	N
ICO-132	N	80	80	20	N	P	N	N	N
ICO-133	N	N	N	N	N	N	N	N	N
ICO-134	N	N	N	N	N	P	N	N	N
ICO-135	N	N	N	N	N	N	N	N	N
ICO-136	N	N	N	N	P	P	N	P	N
ICO-137	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-138	N	N	N	N	N	N	N	N	N
ICO-139	N	N	N	N	P	P	N	N	N
ICO-140	N	N	N	N	P	N	N	N	P
ICO-141	N	N	N	N	P	N	N	N	N
ICO-171	N	N	N	N	N	N	N	N	N
ICO-172	N	N	N	40	P	N	N	N	P
ICO-173	N	N	N	40	N	N	N	N	N
ICO-174	N	N	N	20	N	P	N	P	P
ICO-175	N	N	N	20	N	N	N	N	N
ICO-176	N	N	N	20	P	P	N	P	P
ICO-177	N	N	N	N	N	N	N	N	N
ICO-178	N	N	N	N	N	N	N	N	N
ICO-202	N	N	N	N	N	P	N	P	N
ICO-203	N	N	N	N	N	N	N	N	N
ICO-204	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-205	N	N	N	N	P	P	N	N	N
ICO-206	N	N	N	N	N	N	N	N	N
ICO-207	N	N	N	N	N	N	N	N	N
ICO-208	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-209	N	N	N	20	N	P	N	N	N
ICO-210	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-211	N	N	N	20	P	N	N	P	P
ICO-212	N	N	N	20	N	N	N	N	N
ICO-213	N	N	N	20	N	N	N	N	N
ICO-214	N	N	N	20	N	N	N	N	N
ICO-215	ND	ND	ND	ND	ND	ND	ND	ND	ND

ICO-216	N	N	N	N	N	N	N	N	N
ICO-217	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-218	N	N	N	N	P	N	N	N	N
ICO-219	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-220	N	N	N	N	N	N	N	N	N
ICO-221	N	N	N	20	N	N	N	N	P
ICO-222	N	N	N	20	N	N	N	N	N
ICO-223	N	N	N	40	N	N	N	N	P
ICO-224	N	N	N	40	P	P	N	N	P
ICO-225	N	N	N	40	N	N	N	N	P
ICO-245	N	80	20	80	N	P	N	N	N
ICO-246	N	N	N	N	N	P	N	N	P
ICO-247	N	N	N	20	N	P	N	N	P
ICO-248	N	N	N	20	P	P	N	P	P
ICO-249	N	N	N	N	N	P	N	N	P
ICO-250	N	N	N	N	N	N	N	N	P
SNV-001	N	N	N	N	N	N	N	N	N
SNV-002	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-003	N	N	N	N	N	N	N	N	N
SNV-004	N	N	N	N	N	N	N	N	N
SNV-005	N	N	N	N	N	N	N	N	N
SNV-006	N	N	N	N	N	N	N	N	N
SNV-007	N	N	N	N	N	N	N	N	N
SNV-008	N	N	N	N	N	N	N	N	N
SNV-009	N	N	N	N	N	N	N	N	N
SNV-010	N	N	N	N	N	N	N	N	N
SNV-011	N	N	N	N	N	N	N	N	N
SNV-012	N	N	N	N	N	N	N	N	N
SNV-013	N	N	N	N	N	N	N	N	N
SNV-014	N	N	N	N	N	N	N	N	N
SNV-015	N	N	N	N	N	N	N	N	N
SNV-016	N	N	N	N	N	N	N	N	N
SNV-017	N	N	N	N	N	N	N	N	N
SNV-018	N	N	N	N	N	N	N	N	N
SNV-019	N	N	N	N	N	N	N	N	N
SNV-020	N	N	N	N	N	N	N	N	N
SNV-021	N	N	N	N	N	N	N	N	N
SNV-022	N	N	N	N	N	N	N	N	N
SNV-023	N	N	N	N	N	N	N	N	N
SNV-024	N	N	N	N	N	N	N	N	N
SNV-025	N	N	N	N	N	N	N	N	N
SNV-026	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-027	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-028	N	N	N	N	N	N	N	N	N
SNV-029	N	N	N	N	N	N	N	N	N
SNV-030	N	N	N	N	N	N	N	N	N
SNV-031	N	N	N	N	N	N	N	N	N
SNV-032	N	N	N	N	N	N	N	N	N
SNV-033	N	N	N	N	N	N	N	N	N
SNV-037	N	N	N	N	N	N	N	N	N
SNV-041	N	N	N	N	N	N	N	N	N
SNV-058	N	N	N	N	N	N	N	N	N
SNV-060	N	N	N	N	N	N	N	N	N
SNV-061	N	N	N	N	N	N	N	N	N
SNV-062	N	N	N	N	N	N	N	N	N
SNV-063	ND	ND	ND	ND	ND	ND	ND	ND	ND

SNV-064	N	N	N	N	N	N	N	N	N
SNV-065	N	N	N	N	N	N	N	N	N
SNV-066	N	N	N	N	N	N	N	N	N
SNV-067	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-068	N	N	N	N	N	N	N	N	N
SNV-069	N	N	N	N	N	N	N	N	N
SNV-070	N	N	N	20	N	N	N	N	N
SNV-071	N	N	N	N	N	N	N	N	N
SNV-072	N	N	N	N	N	N	N	N	N
SNV-073	N	N	N	N	N	N	N	N	N
SNV-074	N	N	N	N	N	N	N	N	N
SNV-075	N	N	N	N	N	N	N	N	N
SNV-076	N	N	N	N	N	N	N	N	N
SNV-077	N	N	N	N	N	N	N	N	N
SNV-078	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-079	N	N	N	N	N	N	N	N	N
SNV-080	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-081	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-082	N	N	N	N	N	N	N	N	N
SNV-083	N	N	N	N	N	N	N	N	N
SNV-084	N	N	N	N	N	N	N	N	N
SNV-085	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-086	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-087	N	N	N	N	N	N	N	N	N
SNV-088	N	N	N	N	N	N	N	N	N
SNV-089	N	N	N	N	N	N	N	N	N
SNV-090	N	N	N	N	N	N	N	N	N
SNV-091	N	N	N	N	N	N	N	N	N
SNV-092	N	N	N	N	N	N	N	N	N
SNV-093	N	N	N	N	N	N	N	N	N
SNV-094	N	N	N	N	N	N	N	N	N
SNV-095	N	N	N	N	N	N	N	N	N
SNV-099	N	N	N	20	N	N	N	N	N
SNV-100	N	N	N	N	N	N	N	N	N
SNV-101	N	N	N	N	N	N	N	N	N
SNV-102	N	N	N	N	N	N	N	N	N
SNV-103	N	N	N	N	N	N	N	N	N
SNV-104	N	N	N	N	N	N	N	N	N
SNV-105	N	N	N	N	N	N	N	N	N
SNV-106	N	N	N	N	N	N	N	N	N
SNV-107	N	N	N	N	N	N	N	N	N

TARGET AREA: SERRA DO NAVIO - 3rd sample

CODE	SL	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
ICO-001	N	N	N	N	N	N	N	N	N
ICO-002	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-003	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-004	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-005	N	160	N	N	N	N	N	N	N
ICO-006	N	20	N	20	N	N	N	N	N
ICO-007	N	N	N	N	N	N	N	N	N
ICO-008	N	N	N	N	N	N	N	P	N
ICO-009	N	N	N	N	N	N	P	N	N
ICO-010	N	N	N	N	N	N	N	N	N
ICO-011	N	N	N	N	N	N	P	N	N
ICO-012	N	N	N	40	N	N	N	N	N
ICO-013	N	N	N	N	N	N	N	N	N
ICO-014	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-015	N	N	N	20	N	N	N	N	N
ICO-016	N	N	N	320	N	N	N	N	N
ICO-017	N	N	N	N	N	N	N	N	N
ICO-018	N	N	N	20	N	N	N	N	N
ICO-019	N	N	N	N	N	N	P	P	N
ICO-020	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-021	N	N	N	N	N	N	N	N	N
ICO-022	N	N	N	N	N	N	N	N	N
ICO-023	N	N	N	20	N	N	N	N	N
ICO-024	N	N	N	N	N	N	N	N	N
ICO-025	N	N	N	20	N	N	N	N	N
ICO-026	N	N	N	20	N	N	N	N	N
ICO-027	N	N	N	N	N	N	N	N	N
ICO-028	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-029	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-030	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-031	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-032	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-033	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-034	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-035	N	N	N	20	N	N	N	N	N
ICO-036	N	40	N	N	N	N	N	N	N
ICO-037	N	N	N	N	N	N	P	P	N
ICO-038	N	N	N	N	N	N	N	N	N
ICO-039	N	N	N	20	N	N	N	N	N
ICO-040	N	N	N	40	P	N	N	N	N
ICO-041	N	N	N	N	P	N	P	N	N
ICO-042	N	20	N	N	N	N	N	N	N
ICO-043	N	20	N	N	N	N	N	P	P
ICO-044	N	N	N	20	N	N	N	N	N
ICO-045	N	N	N	N	N	N	N	N	N
ICO-046	N	N	N	N	N	N	N	P	N
ICO-047	N	N	N	N	N	N	N	N	N
ICO-048	N	N	N	N	N	N	N	N	N
ICO-049	N	N	N	N	N	N	N	N	N
ICO-050	N	40	N	N	N	N	N	N	N
ICO-051	N	N	N	N	N	N	N	N	N

ICO-052	N	N	N	20	P	N	N	N	N
ICO-053	N	N	20	20	N	N	N	N	N
ICO-054	N	80	N	N	N	N	N	N	N
ICO-055	N	N	N	N	N	N	N	N	N
ICO-056	N	N	N	20	N	N	N	P	N
ICO-057	N	N	N	N	N	N	N	N	N
ICO-058	N	40	N	20	N	N	N	N	N
ICO-059	N	N	N	20	N	P	N	N	N
ICO-060	N	N	N	N	N	N	N	P	N
ICO-061	N	N	N	20	N	N	N	N	N
ICO-062	N	N	N	20	N	N	N	N	N
ICO-063	N	N	N	20	N	N	N	N	N
ICO-064	N	20	80	20	N	N	N	N	N
ICO-065	N	N	N	20	N	N	N	N	N
ICO-066	N	20	N	20	N	N	N	P	N
ICO-067	N	N	N	N	N	N	P	N	P
ICO-068	N	20	N	20	N	P	P	N	N
ICO-069	N	N	N	20	N	N	N	N	N
ICO-070	N	N	N	N	N	N	N	N	N
ICO-071	N	N	N	N	N	N	N	N	N
ICO-072	N	N	N	20	N	N	N	P	P
ICO-073	N	20	N	20	N	N	N	N	N
ICO-074	N	N	N	N	N	N	N	N	P
ICO-075	N	N	N	N	N	N	N	N	P
ICO-076	N	N	N	N	N	N	N	N	N
ICO-077	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-078	N	40	N	20	N	N	P	N	N
ICO-079	N	N	N	N	N	N	N	N	N
ICO-080	N	N	N	N	N	N	N	N	P
ICO-081	N	N	N	N	N	N	N	N	N
ICO-082	N	N	N	N	N	N	N	N	N
ICO-083	N	N	N	N	N	N	N	N	N
ICO-084	N	N	N	N	N	N	N	N	N
ICO-085	N	N	N	N	N	N	N	N	N
ICO-086	N	N	N	N	N	N	N	N	N
ICO-087	N	N	N	N	N	N	N	N	N
ICO-088	N	N	N	N	N	N	N	N	N
ICO-089	N	N	N	N	N	N	P	N	N
ICO-090	N	N	N	N	N	N	N	N	N
ICO-091	N	N	N	N	N	N	N	N	N
ICO-092	N	N	N	N	N	N	N	N	N
ICO-093	N	N	N	N	N	N	N	P	N
ICO-094	N	N	N	N	N	N	N	N	N
ICO-095	N	N	N	N	N	N	N	N	N
ICO-096	N	N	N	N	N	N	N	N	N
ICO-097	N	N	N	N	P	N	N	N	N
ICO-098	N	N	N	N	N	N	N	N	N
ICO-099	N	N	N	N	N	N	N	N	N
ICO-100	N	N	N	N	N	N	N	N	N
ICO-101	N	N	N	N	P	N	N	N	N
ICO-102	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-103	N	N	N	N	P	N	N	N	N
ICO-104	N	N	N	N	N	N	N	N	P
ICO-105	N	N	N	N	P	N	N	N	N
ICO-106	N	N	N	N	P	N	N	N	N
ICO-107	ND	ND	ND	ND	ND	ND	ND	ND	ND

ICO-108	N	N	N	N	N	P	N	N	N
ICO-109	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-110	N	N	N	N	N	N	N	N	N
ICO-111	N	N	N	N	N	N	N	N	N
ICO-112	N	N	N	N	N	N	N	N	N
ICO-113	N	N	N	N	N	N	N	N	N
ICO-114	N	N	N	N	N	N	N	N	N
ICO-115	N	N	N	N	P	N	N	N	P
ICO-116	N	N	N	N	P	N	N	N	N
ICO-117	N	N	N	N	N	N	N	N	N
ICO-118	N	N	N	N	N	N	N	N	N
ICO-119	N	N	N	N	P	P	P	P	P
ICO-120	N	N	N	N	N	N	N	N	N
ICO-121	N	N	N	N	N	N	N	N	N
ICO-122	N	N	N	N	P	N	N	N	N
ICO-123	N	N	N	N	N	N	N	N	N
ICO-124	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-125	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-126	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-127	N	N	N	N	N	N	N	N	N
ICO-128	N	N	N	N	N	N	N	N	N
ICO-129	N	N	N	N	N	N	N	N	N
ICO-130	N	N	N	N	N	N	N	N	N
ICO-131	N	N	N	N	N	N	N	N	N
ICO-132	N	N	N	N	P	P	N	N	P
ICO-133	N	N	N	N	N	N	N	N	N
ICO-134	N	N	N	N	N	N	N	N	N
ICO-135	N	N	N	N	N	N	N	N	N
ICO-136	N	N	N	N	P	P	N	P	N
ICO-137	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-138	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-139	N	N	N	20	N	N	N	N	N
ICO-140	N	N	N	N	N	N	N	N	N
ICO-141	N	N	N	N	N	N	N	N	P
ICO-171	N	N	N	N	N	N	N	N	N
ICO-172	N	N	N	N	P	P	N	P	N
ICO-173	N	N	N	N	N	P	N	N	N
ICO-174	N	N	N	N	N	N	N	N	N
ICO-175	N	N	N	N	N	N	N	N	N
ICO-176	N	N	N	N	N	N	N	N	P
ICO-177	N	N	N	20	P	P	N	N	N
ICO-178	N	N	N	N	N	N	N	N	N
ICO-202	N	N	N	N	N	N	N	N	N
ICO-203	N	N	N	N	N	N	N	P	N
ICO-204	N	N	N	20	N	N	N	N	N
ICO-205	N	N	N	N	N	N	N	N	N
ICO-206	N	N	N	N	N	N	N	N	N
ICO-207	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-208	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-209	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-210	N	N	N	20	N	N	N	N	N
ICO-211	N	N	N	N	N	N	N	N	N
ICO-212	N	N	N	N	N	N	N	N	N
ICO-213	N	N	N	20	N	N	N	N	N
ICO-214	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-215	N	N	N	N	P	N	N	N	P

ICO-216	N	N	N	N	N	N	N	N	N
ICO-217	N	N	N	N	N	N	N	P	N
ICO-218	N	N	N	N	N	P	N	N	N
ICO-219	ND	ND	ND	ND	ND	ND	ND	ND	ND
ICO-220	N	N	N	N	N	N	N	N	P
ICO-221	N	N	N	N	N	N	N	N	N
ICO-222	N	N	N	N	N	P	N	N	P
ICO-223	N	N	N	N	N	N	N	N	P
ICO-224	N	N	N	20	P	N	N	N	P
ICO-225	N	N	N	N	N	N	N	N	N
ICO-245	N	N	N	20	N	N	N	N	N
ICO-246	N	N	N	N	P	N	N	N	P
ICO-247	N	N	N	N	N	P	N	N	P
ICO-248	N	N	N	N	N	P	N	N	N
ICO-249	N	N	N	N	N	P	N	N	P
ICO-250	N	N	N	N	N	N	N	N	N
SNV-001	N	N	N	N	N	N	N	N	N
SNV-002	N	N	N	N	N	N	N	N	N
SNV-003	N	N	N	N	N	N	N	N	N
SNV-004	N	N	N	N	N	N	N	N	N
SNV-005	N	N	N	N	N	N	N	N	N
SNV-006	N	N	N	N	N	N	N	N	N
SNV-007	N	N	N	20	N	N	N	N	N
SNV-008	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-009	N	N	N	N	N	N	N	N	N
SNV-010	N	N	N	N	N	N	N	N	N
SNV-011	N	N	N	N	N	N	N	N	N
SNV-012	N	N	N	N	N	N	N	N	N
SNV-013	N	N	N	20	N	N	N	N	N
SNV-014	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-015	N	N	N	N	N	N	N	N	N
SNV-016	N	N	N	N	N	N	N	N	N
SNV-017	N	N	N	N	N	N	N	N	N
SNV-018	N	N	N	N	N	N	N	N	N
SNV-019	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-020	N	N	N	N	N	N	N	N	N
SNV-021	N	N	N	N	N	N	N	N	N
SNV-022	N	N	N	N	N	N	N	N	N
SNV-023	N	N	N	N	N	N	N	N	N
SNV-024	N	N	N	N	N	N	N	N	N
SNV-025	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-026	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-027	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-028	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-029	N	N	N	N	N	N	N	N	N
SNV-030	N	N	N	N	N	N	N	N	N
SNV-031	N	N	N	N	N	N	N	N	N
SNV-032	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-033	N	N	N	N	N	N	N	N	N
SNV-037	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-041	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-058	N	N	N	N	N	N	N	N	N
SNV-060	N	N	N	N	N	N	N	N	N
SNV-061	N	N	N	N	N	N	N	N	N
SNV-062	N	N	N	N	N	N	N	N	N
SNV-063	N	N	N	N	N	N	N	N	N

SNV-064	N	N	N	N	N	N	N	N	N
SNV-065	N	N	N	N	N	N	N	N	N
SNV-066	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-067	N	N	N	N	N	N	N	N	N
SNV-068	N	N	N	N	N	N	N	N	N
SNV-069	N	N	N	N	N	N	N	N	N
SNV-070	N	N	N	N	N	N	N	N	N
SNV-071	N	N	N	N	N	N	N	N	N
SNV-072	N	N	N	N	N	N	N	N	N
SNV-073	N	N	N	N	N	N	N	N	N
SNV-074	N	N	N	N	N	N	N	N	N
SNV-075	N	N	N	N	N	N	N	N	N
SNV-076	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-077	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-078	N	N	N	N	N	N	N	N	N
SNV-079	N	N	N	N	N	N	N	N	N
SNV-080	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-081	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-082	N	N	N	N	N	N	N	N	N
SNV-083	N	N	N	N	N	N	N	N	N
SNV-084	N	N	N	N	N	N	N	N	N
SNV-085	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-086	ND	ND	ND	ND	ND	ND	ND	ND	ND
SNV-087	N	N	N	N	N	N	N	N	N
SNV-088	N	N	N	N	N	N	N	N	N
SNV-089	N	N	N	N	N	N	N	N	N
SNV-090	N	N	N	N	N	N	N	N	N
SNV-091	N	N	N	N	N	N	N	N	N
SNV-092	N	N	N	N	N	N	N	N	N
SNV-093	N	N	N	N	N	N	N	N	N
SNV-094	N	N	N	N	N	N	N	N	N
SNV-095	N	N	N	N	N	N	N	N	N
SNV-099	N	N	N	N	N	N	N	N	N
SNV-100	N	N	N	N	N	N	N	N	N
SNV-101	N	N	N	N	N	N	N	N	N
SNV-102	N	N	N	N	N	N	N	N	N
SNV-103	N	N	N	N	N	N	N	N	N
SNV-104	N	N	N	N	N	N	N	N	N
SNV-105	N	N	N	N	N	N	N	N	N
SNV-106	N	N	N	N	N	N	N	N	N
SNV-107	N	N	N	N	N	N	N	N	N

APPENDIX 8

CONTROL AREA: COLONIA AGUA BRANCA

CODE	S	A	MH	SR	SL	HG	HK	CQUR	CQSE	CHS
1/91	M	4	Y4	1	PV	ND	ND	ND	0	Y
18/91	M	1	N	0	PV	ND	ND	ND	1.2	N
2/91	M	26	Y5	?	N	ND	ND	ND	2.2	Y
3/91	M	15	Y5	?	PV	ND	ND	ND	0	Y
32/92	F	17	YS	2	N	ND	ND	ND	0	Y
33/92	M	46	YS	0	N	ND	ND	ND	1.2	N
34/92	F	5	Y2	1	N	ND	ND	ND	1.1	N
35/92	F	22	Y1	0	N	ND	ND	ND	1.1	Y
4/91	M	17	Y1	1	PV	ND	ND	ND	1.1	Y
46/92	F	5	YS	2	N	ND	ND	ND	1.5	Y
47/92	M	4	Y2	2	N	2500	ND	ND	1.1	Y
51/92	M	16	YS	?	N	ND	ND	ND	0	Y
CAB-001	M	7	Y1	0	N	930	ND	ND	1.4	Y
CAB-002	F	3	N	0	N	1610	ND	ND	1.4	Y
CAB-003	F	15	N	0	N	ND	ND	ND	1.2	Y
CAB-004	F	16	N	0	N	ND	ND	ND	1.4	Y
CAB-005	M	32	Y8	0	N	ND	ND	ND	1.2	Y
CAB-006	M	8	N	0	N	ND	ND	ND	1.4	Y
CAB-007	M	6	N	0	N	1510	ND	ND	1.2	Y
CAB-008	F	4	N	0	N	1840	ND	ND	1.5	Y
CAB-009	F	2	N	0	N	>2600	ND	ND	1.2	Y
CAB-010	F	24	Y1	0	N	ND	ND	ND	1.2	Y
CAB-011	F	4	N	0	N	ND	ND	ND	1.4	Y
CAB-012	F	7	N	0	N	ND	ND	ND	1.3	Y
CAB-013	F	14	N	0	N	ND	ND	ND	1.3	Y
CAB-014	F	20	N	0	N	ND	ND	ND	1.4	Y
CAB-015	M	4	N	0	N	ND	ND	ND	1.4	Y
CAB-016	M	17	Y6	0	N	ND	ND	ND	8.1	Y
CAB-017	F	58	Y1	0	N	ND	ND	ND	1.1	Y
CAB-018	F	18	N	0	N	ND	ND	ND	0	Y
CAB-019	M	9	N	0	N	520	ND	ND	0	Y
CAB-020	M	6	N	0	N	980	ND	ND	0	Y
CAB-021	M	2	N	0	N	1840	ND	ND	0	Y
CAB-022	F	3	Y2	0	N	720	ND	ND	0	Y
CAB-023	M	5	N	0	N	2140	ND	ND	1.2	Y
CAB-024	M	7	N	0	N	375	ND	ND	0	Y
CAB-025	F	25	N	0	N	ND	ND	ND	0	Y
CAB-026	M	5	Y1	0	PV	980	ND	ND	6.8	N
CAB-027	F	4	N	0	N	ND	ND	ND	11	N
CAB-028	M	9	Y1	1	PV	1200	ND	ND	98	N
CAB-029	F	8	N	0	N	ND	ND	ND	68	N
CAB-030	F	11	Y1	0	N	>2500	ND	ND	10	N
CAB-031	F	27	N	0	N	ND	ND	ND	3.1	N
CAB-032	M	2	N	0	N	705	ND	ND	9	N
CAB-033	F	19	N	0	N	ND	ND	ND	9.1	N
CAB-034	M	35	Y4	0	N	ND	ND	ND	6.8	N
CAB-035	F	55	Y1	0	N	ND	ND	ND	2.4	N
CAB-036	F	2	N	0	N	ND	ND	ND	0	Y
CAB-037	M	21	Y2	0	N	ND	ND	ND	2.4	Y
CAB-038	F	7	N	0	N	ND	ND	ND	9.1	N

CAB-039	F	11	Y1	0	N	1490	ND	ND	82	N
CAB-040	F	17	N	0	N	ND	ND	ND	68	N
CAB-041	M	19	Y3	0	PF	ND	ND	ND	4.2	N
CAB-042	F	65	N	0	N	ND	ND	ND	4.2	Y
CAB-043	F	13	N	0	N	ND	ND	ND	2.2	Y
CAB-044	M	16	Y3	0	PV	ND	ND	ND	3.3	Y
CAB-045	M	21	Y4	0	N	ND	ND	ND	2.1	Y
CAB-046	F	20	N	0	N	ND	ND	ND	1.5	Y
CAB-047	F	20	N	0	N	ND	ND	ND	1.5	Y
CAB-048	F	4	N	0	N	1910	ND	ND	2.2	Y
CAB-049	M	14	Y1	0	N	1285	ND	ND	1.6	Y
CAB-050	F	16	Y2	0	N	ND	ND	ND	8	N
CAB-051	F	27	Y2	0	N	ND	ND	ND	2.6	N
CAB-052	F	25	Y3	0	PV	ND	ND	ND	1.3	N
CAB-053	F	4	N	0	N	ND	ND	ND	1.5	N
CAB-054	F	8	Y4	0	PF	980	ND	ND	1.5	N
CAB-055	M	28	Y4	0	PF	ND	ND	ND	1.8	N
CAB-056	M	14	Y3	1	N	ND	ND	ND	1.2	N
CULPF01	F	4	Y1	1	PF	ND	ND	ND	ND	N
ICM 001	M	32	Y1	0	PV	ND	ND	ND	ND	N
ICM 002	M	49	Y2	2	PV	ND	ND	ND	ND	Y
ICO-142	M	23	Y4	0	PF	ND	P	7664	15	Y
ICO-143	F	18	Y4	1	PF	ND	P	160	4	N
ICO-144	F	19	Y4	2	PF	ND	P	220	6.5	N
ICO-145	M	36	Y3	0	N	ND	P	100	1.9	Y
ICO-146	M	8	Y4	0	PV	1040	N	180	2.2	Y
ICO-148	M	14	Y1	0	N	ND	P	420	1.9	Y
ICO-149	F	43	N	0	N	ND	P	106	6	Y
ICO-150	M	3	N	0	N	ND	N	100	6.5	Y
ICO-151	M	2	Y4	0	N	ND	N	100	8.5	Y
ICO-152	F	29	Y2	0	N	ND	P	105	8	Y
ICO-153	M	2	N	0	N	ND	P	113	6	Y
ICO-154	M	24	Y4	0	N	ND	P	627	4.6	Y
ICO-155	F	22	Y4	0	N	ND	P	230	1.3	Y
ICO-156	F	19	Y4	0	N	ND	P	160	10.5	Y
ICO-157	M	9	Y1	0	N	ND	N	102	8.8	Y
ICO-158	F	40	Y2	0	N	ND	P	100	2.9	Y
ICO-159	M	9	Y3	0	PV	945	P	220	1.6	Y
ICO-160	F	5	N	0	N	ND	N	103	2.8	Y
ICO-161	M	4	Y1	0	N	ND	P	86	1.5	Y
ICO-162	F	31	Y1	1	N	ND	ND	ND	6	N
ICO-163	F	12	Y1	1	PF	0	ND	ND	1.8	N
ICO-164	M	4	Y3	2	PF	0	ND	ND	5	N
ICO-165	F	1	Y2	1	PF	ND	ND	ND	6.5	N
ICO-166	F	4	Y5	1	PF	0	ND	ND	3.4	N
ICO-167	F	7	Y3	1	PF	0	ND	ND	10	N
ICO-168	F	9	Y2	1	PF	0	ND	ND	6.5	N
ICO-169	M	11	Y5	1	PF	0	ND	ND	2	N
ICO-170	M	17	YS	1	PF	ND	ND	ND	12.5	N
ICO-179	M	72	Y1	0	N	ND	P	230	1	N
ICO-180	M	74	Y1	0	N	ND	P	90	10	N
ICO-181	M	37	N	0	N	ND	N	95	2.6	N
ICO-182	F	70	Y	0	N	ND	P	ND	1.6	N
ICO-183	F	67	N	0	N	ND	P	ND	2.6	N
ICO-184	F	43	N	0	N	ND	P	ND	4.1	N
ICO-185	F	14	Y1	0	N	ND	N	ND	2	N

ICO-186	F	11	N	0	N	ND	P	700	1.3	N
ICO-187	F	66	N	0	N	ND	N	850	4.2	N
ICO-188	F	33	N	0	N	ND	P	ND	2.5	N
ICO-189	F	8	N	0	N	ND	N	ND	3.0	N
ICO-190	F	12	N	0	N	ND	N	150	1.4	N
ICO-191	M	7	N	0	N	ND	N	180	2.4	N
ICO-192	F	4	Y1	0	N	ND	ND	140	1.5	N
ICO-193	M	3	Y1	0	N	ND	ND	ND	6.5	N
ICO-194	F	14	N	0	N	ND	P	23	1	Y
ICO-195	M	9	N	0	N	ND	N	460	8.5	N
ICO-196	M	5	N	0	N	ND	P	1600	12	N
ICO-197	F	10	N	0	N	ND	N	410	2	N
ICO-198	M	12	N	0	N	ND	N	1050	2.1	N
ICO-199	M	2	N	0	N	ND	ND	50	1.9	N
ICO-200	F	14	Y1	0	N	ND	N	1070	2.6	N
ICO-201	M	44	N	0	N	ND	P	400	3	N
ICO-226	F	41	Y1	0	N	ND	P	4800	9.1	N
ICO-227	F	6	N	0	N	ND	ND	ND	10	N
ICO-228	F	18	N	0	N	ND	N	49	8	N
ICO-229	F	4	N	0	N	ND	P	2900	8.2	N
ICO-230	F	24	N	0	N	ND	P	1300	8.5	N
ICO-231	F	16	N	0	N	ND	N	5700	6.5	N
ICO-232	M	2	Y4	0	N	1690	N	7500	18	N
ICO-233	F	4	N	0	N	ND	N	2000	6.2	N
ICO-234	F	26	Y4	1	N	ND	N	1200	8.2	N
ICO-235	F	31	N	0	N	ND	P	150	1.5	N
ICO-236	F	9	N	0	N	ND	P	1550	10	N
ICO-237	M	26	Y2	0	N	ND	P	1700	18	N
ICO-238	M	7	N	0	N	ND	P	850	11	N
ICO-239	F	5	N	0	N	ND	N	390	2.7	N
ICO-240	M	4	N	0	N	ND	P	ND	5.9	N
ICO-241	F	2	N	0	N	ND	P	610	6.5	N
ICO-242	F	23	N	0	N	ND	P	ND	3.5	N
ICO-243	M	36	Y1	0	N	ND	N	920	3	N
ICO-244	M	10	Y1	0	N	ND	P	ND	6	N
PF05/91	F	42	Y1	1	PF	ND	ND	ND	0	Y
PF06/91	M	21	Y3	2	PF	ND	ND	ND	0	N
PF07/91	F	16	Y1	0	PF	ND	ND	ND	1.4	Y
PF08/91	F	5	Y3	0	PF	ND	ND	ND	1.6	Y
PF09/91	F	15	Y3	2	PF	ND	ND	ND	1.2	Y
PF10/91	M	35	Y1	0	PF	ND	ND	ND	1.2	N
PF11/91	F	21	N	0	PF	ND	ND	ND	1.4	Y
PF12/91	M	8	Y2	1	PF	ND	ND	ND	1.4	N
PF13/91	M	19	Y2	0	PF	ND	ND	ND	1.3	N
PF14/91	M	22	Y2	0	PF	ND	ND	ND	1.1	N
PF30/92	M	3	Y1	0	PF	ND	ND	ND	3.3	Y
PF37/92	M	62	YS	2	PF	ND	ND	ND	ND	N
PF40/92	F	17	Y8	1	PF	ND	ND	ND	1.6	N
PF42/92	M	31	YS	1	PF	ND	ND	ND	2.8	N
PF52/89	M	17	Y1	0	PF	ND	ND	ND	ND	N
PF54/90	M	30	Y3	2	PF	ND	ND	ND	ND	N
PF56/90	F	29	Y1	1	PF	ND	ND	ND	ND	Y
PF57/90	M	46	Y1	3	PF	ND	ND	ND	ND	Y
PF58/90	M	30	Y1	1	PF	ND	ND	ND	ND	N
PF59/90	F	3	Y1	1	PF	ND	ND	ND	ND	N
PV26/92	M	39	N	0	PF	ND	ND	ND	13	N

PV27/92	M	19	Y6	0	PV	ND	ND	ND	3.5	N
PV28/92	M	38	Y4	2	PV	ND	ND	ND	ND	N
PV36/92	M	38	YS	0	PV	ND	ND	ND	4.9	N
PV39/92	M	30	Y2	0	PV	ND	ND	ND	2.1	Y
PV50/92	M	17	YS	0	PV	ND	ND	ND	4.2	Y
SNV-113	M	41	Y1	1	N	ND	ND	ND	ND	N
SNV-118	M	1	Y1	1	N	ND	ND	ND	ND	N
SNV-119	F	28	Y1	0	N	ND	ND	ND	ND	N
SNV-122	M	15	Y1	0	N	ND	ND	ND	1.1	N
SNV-124	M	21	Y3	2	PF	ND	ND	ND	ND	Y
SNV-125	M	31	Y2	1	PF	ND	ND	ND	ND	N
SNV-126	M	37	Y1	0	N	ND	ND	ND	ND	Y
SNV-127	M	25	Y3	2	PV	ND	ND	ND	1.2	Y
SNV-34	F	23	Y2	1	MX	ND	ND	ND	10	N
SNV-35	F	14	Y1	0	N	ND	ND	ND	0	N
SNV-36	F	35	Y2	1	N	ND	ND	ND	7.5	N
SNV-38	F	30	Y	0	N	ND	ND	ND	8.2	N
SNV-39	F	9	Y2	0	N	ND	ND	ND	5.4	N
SNV-40	F	8	N	0	N	450	ND	ND	8.5	N
SNV-42	M	63	N	0	N	ND	ND	ND	7	N
SNV-43	M	26	Y3	2	N	ND	ND	ND	2.6	N
SNV-44	F	22	Y1	0	N	ND	ND	ND	3	Y
SNV-45	M	51	N	0	N	ND	ND	ND	ND	Y
SNV-46	F	49	N	0	N	ND	ND	ND	ND	Y
SNV-47	M	19	Y2	1	N	ND	ND	ND	5.2	Y
SNV-48	M	24	Y2	0	N	ND	ND	ND	2.1	Y
SNV-49	M	18	N	0	PF	ND	ND	ND	10.7	Y
SNV-50	F	10	N	0	N	ND	ND	ND	1.5	Y
SNV-51	M	7	N	0	N	ND	ND	ND	1.9	Y
SNV-52	F	27	Y1	1	N	ND	ND	ND	2.7	N
SNV-53	F	7	N	0	N	ND	ND	ND	2.5	N
SNV-54	F	63	Y1	0	N	ND	ND	ND	1.5	Y
SNV-55	M	71	Y1	0	N	ND	ND	ND	1.5	Y
SNV-56	M	36	Y1	0	N	ND	ND	ND	1.1	Y
SNV-57	M	46	Y1	0	N	ND	ND	ND	ND	Y
SNV-59	M	19	Y4	2	N	ND	ND	ND	1.5	N

CONTROL AREA: COLONIA AGUA BRANCA

CODE	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
1/91	640	80	40	N	N	N	N	N
18/91	20	80	N	P	N	N	N	N
2/91	80	N	N	N	N	N	N	N
3/91	80	40	40	N	N	N	N	N
32/92	320	80	N	N	N	N	N	N
33/92	320	N	160	N	N	N	N	N
34/92	N	N	N	P	N	N	N	N
35/92	320	N	20	P	N	N	N	N
4/91	160	80	40	N	N	N	N	N
46/92	80	N	N	N	N	P	N	N
47/92	160	N	N	N	N	N	N	N
51/92	80	N	N	N	N	N	N	N
CAB-001	N	N	N	N	N	N	P	N
CAB-002	N	N	N	N	N	N	P	P
CAB-003	N	N	N	N	N	N	P	N
CAB-004	N	20	N	N	N	N	P	N
CAB-005	640	160	20	N	N	N	N	N
CAB-006	N	N	N	P	N	N	N	N
CAB-007	N	20	N	N	N	N	N	N
CAB-008	N	20	N	N	P	N	P	P
CAB-009	N	N	N	N	N	N	N	N
CAB-010	N	N	N	N	N	N	P	P
CAB-011	N	N	20	N	N	N	P	P
CAB-012	20	N	N	N	N	N	P	P
CAB-013	N	N	N	P	N	N	P	P
CAB-014	N	N	N	N	N	N	P	N
CAB-015	N	N	N	N	P	N	P	P
CAB-016	640	320	20	P	P	N	P	P
CAB-017	640	320	N	N	P	N	P	P
CAB-018	N	N	20	P	P	N	P	N
CAB-019	N	N	N	N	N	N	P	P
CAB-020	N	N	N	P	N	N	P	P
CAB-021	N	N	N	N	N	N	N	N
CAB-022	N	20	20	P	P	N	P	P
CAB-023	N	20	N	N	N	N	P	N
CAB-024	N	N	N	N	N	N	P	P
CAB-025	N	N	N	N	P	N	P	P
CAB-026	N	N	N	N	N	N	P	P
CAB-027	N	N	N	N	N	N	P	P
CAB-028	640	320	N	P	P	N	P	P
CAB-029	N	N	N	P	P	N	P	P
CAB-030	N	N	20	N	N	N	P	N
CAB-031	N	N	20	N	P	N	P	P
CAB-032	N	N	N	N	N	N	N	N
CAB-033	20	N	20	N	N	N	N	N
CAB-034	640	80	640	N	P	N	P	N
CAB-035	20	N	20	N	N	N	P	N
CAB-036	N	N	20	N	N	N	N	N
CAB-037	160	40	20	N	N	N	P	N
CAB-038	N	N	N	N	P	N	P	P
CAB-039	N	N	N	N	N	N	P	P
CAB-040	40	20	N	N	P	N	P	N

CAB-041	640	80	20	P	N	N	N	N
CAB-042	20	40	20	N	N	N	N	N
CAB-043	N	N	N	N	N	N	N	N
CAB-044	640	640	80	N	N	N	N	N
CAB-045	640	40	640	N	N	N	N	N
CAB-046	N	N	20	N	N	N	N	N
CAB-047	N	N	20	N	N	N	N	N
CAB-048	N	N	20	N	N	N	N	N
CAB-049	20	N	80	N	N	N	N	N
CAB-050	20	N	20	P	N	N	N	N
CAB-051	N	N	20	N	N	N	N	N
CAB-052	N	80	20	N	P	N	N	N
CAB-053	N	N	N	N	N	N	N	N
CAB-054	80	20	N	N	N	N	N	N
CAB-055	640	N	320	P	N	P	N	P
CAB-056	320	80	N	N	P	N	N	N
CULTPF01	ND	ND	ND	ND	ND	ND	ND	ND
ICM 001	20	20	ND	ND	ND	ND	ND	ND
ICM 002	20	20	ND	ND	ND	ND	ND	ND
ICO-142	640	N	20	N	N	N	N	N
ICO-143	640	20	20	N	N	N	N	N
ICO-144	N	N	80	P	P	N	N	N
ICO-145	640	20	80	N	N	N	N	N
ICO-146	N	N	40	N	N	N	N	P
ICO-148	N	N	20	N	N	N	N	N
ICO-149	N	N	N	N	N	N	N	N
ICO-150	N	N	N	P	N	N	N	N
ICO-151	N	N	20	P	N	N	N	N
ICO-152	20	N	20	N	P	N	N	N
ICO-153	N	N	N	N	N	N	N	N
ICO-154	160	N	20	N	N	N	N	N
ICO-155	640	20	20	N	N	N	N	N
ICO-156	640	80	20	N	N	N	N	N
ICO-157	N	20	N	N	N	N	N	N
ICO-158	N	40	N	P	N	N	N	N
ICO-159	N	40	N	N	N	N	N	N
ICO-160	N	N	160	N	N	N	N	N
ICO-161	N	80	N	P	N	N	N	P
ICO-162	160	40	20	P	P	P	N	P
ICO-163	160	N	640	P	P	N	N	P
ICO-164	80	N	20	N	P	N	N	N
ICO-165	80	N	20	P	P	N	N	P
ICO-166	80	N	40	P	P	N	P	N
ICO-167	160	N	20	P	N	N	N	P
ICO-168	160	20	20	P	N	N	N	N
ICO-169	160	N	20	N	N	N	N	N
ICO-170	80	160	320	P	N	N	N	P
ICO-179	20	N	20	P	P	N	N	N
ICO-180	20	N	20	P	P	P	N	N
ICO-181	N	N	N	P	N	N	N	P
ICO-182	N	N	20	N	N	N	N	N
ICO-183	20	N	20	P	N	N	N	N
ICO-184	20	N	20	N	P	N	N	N

ICO-185	20	N	20	P	N	N	N	N
ICO-186	N	N	20	P	N	N	N	N
ICO-187	N	N	20	P	N	N	N	N
ICO-188	N	N	20	N	N	N	N	N
ICO-189	N	N	20	P	N	N	N	N
ICO-190	N	N	20	P	N	N	N	N
ICO-191	N	N	20	P	N	N	N	N
ICO-192	N	N	20	N	N	N	N	N
ICO-193	N	N	20	N	N	N	N	N
ICO-194	N	N	20	N	N	N	N	N
ICO-195	N	N	N	N	P	N	N	P
ICO-196	N	N	N	P	P	N	N	N
ICO-197	N	N	20	N	P	N	N	N
ICO-198	N	N	20	P	P	N	P	P
ICO-199	N	20	20	N	N	N	N	P
ICO-200	160	20	20	P	N	N	N	P
ICO-201	20	N	20	N	P	N	N	P
ICO-226	N	N	N	P	N	N	N	N
ICO-227	N	N	N	N	N	N	P	P
ICO-228	N	N	20	N	N	N	N	N
ICO-229	N	N	20	P	P	N	N	P
ICO-230	N	N	N	P	P	N	N	P
ICO-231	N	N	20	P	N	N	N	N
ICO-232	N	N	20	N	N	N	N	N
ICO-233	N	N	20	N	N	N	N	N
ICO-234	N	N	N	N	N	N	N	N
ICO-235	N	N	N	P	P	N	N	P
ICO-236	N	N	N	N	N	N	N	N
ICO-237	20	N	20	N	P	N	N	N
ICO-238	N	N	20	N	P	N	N	N
ICO-239	N	N	20	N	N	N	N	N
ICO-240	N	N	20	N	P	N	N	P
ICO-241	N	N	20	P	P	N	N	P
ICO-242	N	N	20	P	P	N	N	N
ICO-243	320	20	20	N	N	N	N	P
ICO-244	N	20	20	P	N	N	N	N
PF 05/91	160	160	80	N	N	N	P	P
PF 06/91	80	N	N	N	N	N	N	N
PF 07/91	320	160	N	N	P	N	P	P
PF 08/91	320	N	N	P	N	N	P	N
PF 09/91	320	20	160	P	P	N	P	N
PF 10/91	320	N	320	N	N	N	P	N
PF 11/91	160	80	80	N	P	N	P	P
PF 12/91	160	40	80	N	P	N	P	P
PF 13/91	320	20	160	P	P	N	P	N
PF 14/91	80	N	160	P	P	N	P	P
PF 30/92	320	320	N	P	N	N	N	N
PF 37/92	160	80	80	N	N	N	N	N
PF 40/92	80	N	N	N	N	N	N	N
PF 42/92	320	N	N	P	N	N	N	N
PF 52/89	N	20	N	N	N	N	N	N
PF 54/90	N	N	N	N	N	N	N	N
PF 56/90	320	40	40	N	N	N	N	N

PF 57/90	640	N	N	N	N	N	N	N
PF 58/90	320	80	N	N	N	N	N	N
PF 59/90	640	80	20	N	N	N	N	N
PV 26/92	N	160	N	P	P	N	N	N
PV 27/92	320	80	N	P	P	N	N	N
PV 28/92	N	N	20	N	N	N	N	N
PV 36/92	160	80	20	N	N	N	N	N
PV 39/92	80	80	160	P	N	N	N	N
PV 50/92	80	80	N	P	P	P	N	N
SNV-113	40	N	N	N	N	N	N	N
SNV-118	N	N	N	N	N	N	N	N
SNV-119	N	N	N	N	N	N	N	N
SNV-122	N	N	N	P	N	N	P	N
SNV-124	320	N	N	N	N	N	N	N
SNV-125	160	N	N	N	N	N	N	N
SNV-126	40	N	N	N	N	N	N	N
SNV-127	20	80	N	N	N	N	N	N
SNV-34	320	N	80	P	P	N	N	N
SNV-35	20	N	N	N	N	N	N	N
SNV-36	320	160	160	P	P	N	P	N
SNV-38	N	N	N	P	N	N	N	N
SNV-39	N	N	N	N	N	N	N	N
SNV-40	N	N	N	P	N	N	N	N
SNV-42	N	N	N	P	N	N	N	N
SNV-43	N	N	N	P	P	N	N	N
SNV-44	20	N	N	N	N	N	N	N
SNV-45	N	20	N	N	N	N	N	N
SNV-46	N	N	N	N	N	N	N	N
SNV-47	N	N	N	N	P	N	N	N
SNV-48	N	N	N	N	P	N	N	N
SNV-49	160	N	80	P	P	N	N	N
SNV-50	N	N	N	P	P	N	N	N
SNV-51	40	N	N	N	N	N	N	N
SNV-52	N	N	N	P	N	N	N	N
SNV-53	N	N	N	P	N	N	N	N
SNV-54	N	N	N	N	N	N	N	N
SNV-55	20	N	N	N	N	N	N	N
SNV-56	N	N	N	N	N	N	N	N
SNV-57	N	N	N	N	N	N	N	N
SNV-59	N	N	N	P	N	N	P	P

APPENDIX 9

CONTROL AREA: PORTO TEREZINHA

CODE	S	A	MH	SR	SL	HG	HK	CQUR	CQSE	CHS
31/92	M	61	Y2	0	N	ND	ND	ND	1.3	Y
38/92	F	3	Y1	0	N	1885	ND	ND	1.4	Y
44/92	F	18	Y2	0	N	ND	ND	ND	1.2	Y
PF16/91	F	17	Y4	3	PF	ND	ND	ND	1.5	Y
PF29/92	M	30	Y2	2	PF	ND	ND	ND	1.5	Y
PF48/92	M	25	Y1	0	PF	ND	ND	ND	0	Y
PF50/89	F	23	Y3	2	PF	ND	P	980	2.8	N
PF51/89	M	4	Y2	2	PF	ND	ND	ND	ND	N
PT-001	M	4	N	0	N	2310	P	780	9.6	Y
PT-002	F	2	N	0	N	ND	P	750	1.7	Y
PT-003	F	7	N	0	N	ND	N	230	2	Y
PT-004	F	23	Y1	0	N	ND	P	900	1.1	Y
PT-005	F	11	N	0	N	ND	P	680	2.2	Y
PT-006	F	59	N	0	N	ND	N	610	4.2	Y
PT-007	M	20	Y6	3	N	ND	P	1200	5.1	Y
PT-008	F	19	N	0	N	ND	P	740	2.3	Y
PT-009	M	3	N	0	N	ND	N	800	1.5	Y
PT-011	F	23	Y2	1	N	ND	P	1000	2	Y
PT-012	M	19	Y5	1	N	ND	P	580	ND	Y
PT-013	M	12	Y1	0	N	1575	N	880	1.4	Y
PT-014	F	6	N	0	N	ND	P	800	1.5	Y
PT-015	M	8	Y1	0	N	2240	N	850	6.2	Y
PT-016	F	40	Y1	0	N	ND	P	490	2.8	Y
PT-017	M	46	Y1	0	N	ND	N	750	1.5	Y
PT-018	M	52	Y6	2	N	ND	P	850	1.5	Y
PT-019	F	13	N	0	N	ND	P	550	1.2	Y
PT-020	F	12	N	0	N	ND	P	490	1.7	Y
PT-021	F	25	Y1	0	N	ND	N	1000	2.4	Y
PT-022	F	31	N	0	N	ND	ND	ND	2.2	Y
PT-023	M	9	N	0	N	ND	N	310	1.5	Y
PT-024	M	13	Y1	0	PV	<250	P	1000	6	Y
PT-025	M	7	N	0	N	ND	N	270	1.7	Y
PT-026	F	6	N	0	N	ND	N	540	2.2	Y
PT-027	F	38	Y1	0	N	ND	P	620	ND	Y
PT-028	F	8	N	0	N	ND	N	1150	6.7	Y
PT-029	F	2	N	0	N	ND	N	1000	2.8	Y
PT-030	M	5	N	0	N	ND	P	1300	1.4	Y
PT-031	F	24	N	0	N	ND	P	750	1.5	Y
PT-032	M	9	Y1	0	N	2500	P	740	1.2	Y
PT-033	F	28	Y6	2	N	ND	P	1200	1.4	Y
PT-034	M	23	Y4	1	PF	ND	N	1350	1.6	Y
PT-035	F	21	N	0	N	ND	P	1000	1.5	Y
PT-036	M	14	N	0	N	ND	P	700	1.5	Y
PT-037	M	24	Y4	2	N	ND	P	3900	ND	Y
PT-038	M	48	Y4	1	N	ND	P	1100	2.1	Y
PT-039	M	32	Y2	0	N	ND	ND	ND	2.4	N
PT-040	M	20	Y4	3	N	ND	P	750	ND	Y
PT-041	F	12	N	0	N	ND	P	460	2	Y
PT-042	F	57	Y1	0	N	ND	P	4400	ND	Y
PT-043	F	49	Y2	0	N	ND	P	750	1.9	Y

PT-044	F	7	N	0	N	ND	P	390	1.5	Y
PT-045	F	25	N	0	N	ND	P	380	1.8	Y
PT-046	M	4	N	0	N	ND	P	470	1.3	Y
PT-047	F	27	Y4	2	N	ND	P	880	1.4	Y
PT-048	F	11	N	0	N	ND	P	700	1.4	Y
PT-049	M	10	N	0	N	ND	P	690	1.5	Y
PT-050	F	28	N	0	N	ND	N	460	1.5	Y
PT-051	M	4	N	0	N	2600	P	1200	1.3	Y
PT-052	M	2	N	0	N	ND	ND	ND	1.5	Y
PT-053	F	6	N	0	N	ND	ND	ND	1.5	Y
PT-054	M	4	N	0	N	ND	P	1000	1.3	Y
PT-055	M	45	Y1	0	N	ND	P	810	2.4	Y
PT-056	F	25	N	0	N	ND	P	480	ND	Y
PT-057	F	9	N	0	N	ND	P	1200	1.5	Y
PT-058	F	20	N	0	N	ND	P	980	1.3	Y
PT-059	F	9	N	0	N	ND	P	720	1.2	Y
PT-060	F	14	N	0	N	ND	P	850	1.2	Y
PT-061	F	27	N	0	N	ND	P	2000	ND	Y
PT-062	F	12	N	0	N	ND	P	750	1.5	Y
PT-063	M	5	N	0	N	ND	P	900	1.8	Y
PT-064	F	7	N	0	N	ND	P	1400	1.2	Y
PT-065	M	13	N	0	N	ND	P	990	1.3	Y
PT-066	M	5	N	0	N	ND	N	860	1.3	Y
PT-067	M	2	N	0	N	2600	ND	ND	1.5	Y
PT-068	F	5	N	0	N	ND	ND	ND	1.2	Y
PT-069	F	3	N	0	N	ND	ND	ND	1.5	Y
PT-070	F	23	N	0	N	ND	N	650	1.5	Y
PT-071	M	8	Y1	0	N	ND	P	2900	ND	Y
PT-072	M	11	Y5	2	N	1425	N	1200	1.3	Y
PT-073	F	9	N	0	N	ND	N	3500	1.3	Y
PT-074	M	20	Y6	2	N	ND	N	360	1.2	Y
PT-075	F	59	Y1	0	N	ND	ND	ND	1.5	Y
PT-076	F	20	N	0	N	ND	N	500	1.2	Y
PT-077	M	43	YS	1	N	ND	P	1200	1.3	Y
PT-078	M	17	Y4	1	N	ND	P	690	1.3	Y
PT-079	F	6	N	0	N	ND	N	630	1.3	Y
PT-080	M	13	N	0	N	ND	N	450	1.6	Y
PT-081	M	4	N	0	N	ND	P	890	ND	Y
PT-082	F	43	N	0	N	ND	N	490	1.2	Y
PT-083	F	17	N	0	N	ND	N	750	1.5	Y
PT-084	M	60	Y1	0	N	ND	N	680	1.4	Y
PT-085	M	17	Y3	1	N	ND	P	1100	1.2	Y
PT-086	M	17	Y1	0	N	ND	P	920	1.2	Y
PT-087	F	28	N	0	N	ND	P	1000	0	Y
PT-088	F	9	N	0	N	ND	N	2100	1.2	Y
PT-089	F	7	N	0	N	ND	N	210	1.4	Y
PT-090	F	3	N	0	N	ND	N	660	0	Y
PT-091	M	5	N	0	N	ND	N	410	1.3	Y
PT-092	F	32	N	0	N	ND	P	1300	1.5	Y
PT-093	M	32	Y1	0	N	ND	ND	ND	1.5	Y
PT-094	F	12	Y1	0	N	1360	N	460	1.1	Y
PT-095	M	14	N	0	N	ND	N	600	1.5	Y
PT-096	M	8	N	0	N	ND	P	750	1.2	Y
PT-097	M	14	Y4	2	N	ND	P	570	ND	Y
PT-098	M	4	Y4	2	N	2500	ND	ND	2.4	Y
PT-099	F	6	N	0	N	ND	N	790	1.2	Y

PT-100	M	11	Y1	0	N	ND	N	820	0	Y
PT-101	F	11	N	0	N	ND	N	700	1.5	Y
PT-102	F	12	N	0	N	880	N	850	1.5	Y
PT-103	F	6	Y1	1	N	ND	ND	ND	ND	Y
PT-104	F	14	N	0	N	670	N	850	1.3	Y
PT-105	M	11	N	0	N	ND	N	900	1.4	Y
PT-106	F	8	N	0	N	ND	N	820	1.3	Y
PT-107	F	23	Y4	2	PF	ND	P	980	2.8	Y
PT-108	F	10	N	0	N	ND	N	810	1.3	Y
PT-109	M	46	N	0	N	ND	P	600	1.3	Y
PT-110	F	9	N	0	N	ND	N	460	2.4	Y
PT-111	F	7	N	0	N	ND	P	620	1.6	Y
PT-112	M	27	Y1	0	N	ND	P	1200	1.5	Y
PT-113	M	5	N	0	N	ND	N	0	1.8	Y
PT-114	F	28	N	0	N	ND	N	820	1.2	Y
PT-115	M	4	N	0	N	ND	N	660	1.5	Y
PT-116	F	3	Y1	1	N	980	N	900	1.3	Y
PT-117	F	44	Y2	0	N	ND	N	900	1.2	Y
PT-118	F	30	N	0	N	ND	P	650	2.3	Y
PT-119	F	20	Y4	2	N	ND	N	600	0	Y
PT-120	M	2	N	0	N	ND	ND	ND	0	Y
PT-121	F	37	N	0	N	ND	ND	ND	1.5	Y
PT-122	F	19	Y1	0	N	ND	ND	ND	1.5	Y
PT-123	M	49	Y4	2	N	ND	ND	ND	1.5	Y
PT-124	F	45	Y4	3	N	ND	ND	ND	1.3	Y
PT-125	M	50	Y1	0	N	ND	ND	ND	1.3	Y
PT-126	M	7	N	0	N	1530	ND	ND	1.3	Y
PT-127	F	13	Y1	0	N	<250	ND	ND	0	Y
PT-128	M	12	N	0	N	ND	ND	ND	1.2	Y
PT-129	M	7	N	0	N	2500	ND	ND	1.5	Y
PT-130	F	11	N	0	N	ND	ND	ND	1.3	Y
PT-131	M	6	N	0	N	ND	ND	ND	1.5	Y
PT-132	F	30	Y1	0	N	ND	ND	ND	1.6	Y
PT-133	F	14	N	0	N	ND	ND	ND	1.4	Y
PT-134	F	12	N	0	N	660	ND	ND	1.5	Y
PT-135	M	9	N	0	N	890	ND	ND	1.3	Y
PT-136	M	9	N	0	N	ND	ND	ND	1.3	Y
PT-137	M	50	Y4	0	N	ND	ND	ND	1.1	Y
PT-138	F	36	Y1	0	N	ND	ND	ND	1.5	Y
PT-139	M	18	Y4	1	N	ND	ND	ND	1.3	Y
PT-140	F	12	N	0	N	ND	ND	ND	1.4	Y
PT-141	M	9	N	0	N	ND	ND	ND	1.2	Y
PT-142	M	7	N	0	N	ND	ND	ND	1.5	Y
PT-143	F	2	N	0	N	ND	ND	ND	1.5	Y
PT-144	M	25	Y4	2	N	ND	ND	ND	0	Y
PT-145	F	12	Y2	0	N	680	ND	ND	1.1	Y
PT-146	M	7	Y1	0	N	250	ND	ND	1.5	Y
PT-147	F	12	N	0	N	ND	ND	ND	1.1	Y
PT-148	F	7	N	0	N	ND	ND	ND	1.3	Y
PV25/92	F	25	Y3	0	PV	ND	ND	ND	ND	Y
PV49/92	F	20	N	1	PV	ND	ND	ND	ND	Y
SNV-123	M	25	Y3	1	N	ND	ND	ND	ND	N
SNV02/91	M	23	Y4	2	N	ND	ND	ND	0	Y
SNV07/89	M	43	Y1	0	N	ND	ND	ND	ND	N

CODE	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
31/92	160	640	20	N	P	N	N	P
38/92	160	80	160	N	N	N	N	P
44/92	320	80	N	N	N	N	N	N
PF16/91	640	80	ND	P	P	N	P	P
PF29/92	320	80	N	N	N	N	P	P
PF48/92	640	80	N	N	P	N	P	N
PF50/89	40	40	80	N	N	N	N	N
PF51/89	160	40	N	N	N	N	N	N
PT-001	N	N	N	N	N	N	N	N
PT-002	N	N	N	N	N	N	N	N
PT-003	N	N	320	N	N	N	N	N
PT-004	N	N	640	N	N	N	N	N
PT-005	N	20	20	N	N	N	N	N
PT-006	20	N	160	N	N	N	N	N
PT-007	160	N	20	N	N	N	N	N
PT-008	N	N	20	N	N	N	N	P
PT-009	ND	ND	ND	ND	ND	ND	ND	ND
PT-011	80	N	640	P	N	N	N	P
PT-012	N	N	20	N	N	N	N	N
PT-013	N	N	N	P	N	N	N	N
PT-014	20	N	N	N	N	N	N	P
PT-015	N	N	N	N	N	N	N	N
PT-016	20	N	40	P	N	N	N	N
PT-017	N	20	20	P	N	N	N	N
PT-018	80	N	160	N	N	N	N	N
PT-019	N	N	20	N	N	N	N	N
PT-020	N	N	20	N	N	N	N	N
PT-021	N	N	20	N	N	N	N	N
PT-022	N	N	N	N	N	N	N	N
PT-023	N	N	N	N	N	N	N	N
PT-024	20	80	20	N	N	N	N	N
PT-025	N	N	20	N	N	N	N	N
PT-026	20	N	20	N	N	N	N	N
PT-027	N	N	ND	N	N	N	N	N
PT-028	N	N	20	N	N	N	N	N
PT-029	N	N	20	N	N	P	N	P
PT-030	N	N	20	N	N	N	N	N
PT-031	80	40	20	N	N	N	N	N
PT-032	N	N	20	N	N	N	N	N
PT-033	N	N	20	P	N	N	N	N
PT-034	640	N	640	P	N	N	N	N
PT-035	N	N	N	N	N	N	N	N
PT-036	N	N	20	N	N	N	N	N
PT-037	ND	ND	ND	N	N	N	N	N
PT-038	80	N	N	N	N	N	N	N
PT-039	40	N	20	N	N	N	N	N
PT-040	ND	ND	ND	N	N	N	N	N
PT-041	N	N	N	P	N	N	N	N
PT-042	ND	ND	ND	N	N	N	N	N
PT-043	N	N	20	P	P	N	N	N
PT-044	N	N	20	N	N	N	N	N
PT-045	N	N	20	N	N	N	N	N
PT-046	160	N	N	N	N	N	N	N

PT-047	N	N	20	N	N	N	N	N
PT-048	N	N	20	N	N	N	N	N
PT-049	N	N	N	N	N	N	N	N
PT-050	N	N	N	N	N	N	N	N
PT-051	N	N	20	N	N	N	N	N
PT-052	80	N	20	N	N	N	N	N
PT-053	N	N	N	N	N	N	N	N
PT-054	N	N	20	N	N	N	N	N
PT-055	20	N	20	N	N	N	N	N
PT-056	ND	ND	20	N	N	N	N	N
PT-057	N	N	N	N	N	N	N	N
PT-058	N	20	20	N	N	N	N	N
PT-059	N	N	20	N	N	N	N	N
PT-060	N	N	20	N	N	N	N	N
PT-061	ND	ND	ND	ND	ND	ND	ND	ND
PT-062	N	N	N	N	N	N	N	N
PT-063	N	N	20	N	N	N	N	N
PT-064	N	N	N	N	N	N	N	N
PT-065	N	N	N	P	N	N	N	N
PT-066	N	N	N	N	N	N	N	N
PT-067	N	N	N	P	N	N	N	N
PT-068	N	N	N	N	N	N	N	N
PT-069	N	N	N	N	N	N	N	N
PT-070	N	N	N	N	N	N	N	N
PT-071	ND	ND	ND	ND	ND	ND	ND	ND
PT-072	N	N	ND	ND	ND	ND	ND	ND
PT-073	N	N	20	N	N	N	N	N
PT-074	N	N	N	N	N	N	N	N
PT-075	20	N	20	N	N	N	N	N
PT-076	20	N	N	N	N	N	N	N
PT-077	640	160	20	P	N	N	N	N
PT-078	160	N	20	N	P	N	N	N
PT-079	40	N	80	N	N	N	N	N
PT-080	N	N	20	N	N	N	N	N
PT-081	N	N	N	N	N	N	N	N
PT-082	160	N	20	N	N	N	N	N
PT-083	N	N	20	N	N	N	N	N
PT-084	20	N	N	N	N	N	N	N
PT-085	N	N	N	N	N	N	P	N
PT-086	N	N	N	N	N	N	N	N
PT-087	20	N	N	N	N	N	N	N
PT-088	N	N	20	N	N	N	N	N
PT-089	N	N	N	N	N	N	N	N
PT-090	N	N	N	N	N	N	N	N
PT-091	N	N	N	N	N	N	N	N
PT-092	N	N	N	N	N	N	P	N
PT-093	N	40	20	N	N	N	N	N
PT-094	320	20	N	N	N	N	N	N
PT-095	N	N	20	P	P	P	P	P
PT-096	N	N	20	P	P	N	P	P
PT-097	ND	ND	N	N	N	N	N	N
PT-098	80	N	20	N	N	N	N	N
PT-099	N	N	N	N	N	N	P	N
PT-100	N	N	N	N	N	N	N	N
PT-101	N	N	N	N	N	N	N	N
PT-102	80	40	N	N	N	N	N	N

PT-103	N	N	N	N	N	N	N	N
PT-104	40	N	20	N	N	N	N	P
PT-105	N	N	N	N	N	P	N	N
PT-106	N	N	20	N	N	N	N	N
PT-107	160	N	20	N	N	N	P	N
PT-108	N	N	N	N	N	N	N	N
PT-109	640	40	640	N	N	N	N	N
PT-110	N	N	N	N	N	N	N	N
PT-111	20	N	N	N	N	N	P	N
PT-112	N	N	N	N	N	N	N	N
PT-113	N	N	N	N	N	N	P	P
PT-114	N	N	N	P	N	N	N	N
PT-115	N	N	160	N	N	N	N	N
PT-116	N	N	N	N	N	N	N	N
PT-117	40	N	40	N	N	N	P	N
PT-118	N	N	ND	P	N	N	N	N
PT-119	80	N	20	N	N	N	N	P
PT-120	N	N	N	N	N	N	N	N
PT-121	N	N	N	N	N	N	N	N
PT-122	N	N	N	N	P	N	P	N
PT-123	640	N	640	P	P	N	P	P
PT-124	80	N	20	P	P	N	P	P
PT-125	160	N	20	N	P	N	P	P
PT-126	N	N	N	N	P	N	N	N
PT-127	N	N	20	N	N	N	N	P
PT-128	160	N	320	N	P	N	P	P
PT-129	N	N	N	N	N	N	P	N
PT-130	N	N	20	N	N	N	N	P
PT-131	N	N	N	N	P	N	P	P
PT-132	N	N	N	N	P	N	P	N
PT-133	N	N	N	N	N	N	P	N
PT-134	N	N	N	N	N	N	P	P
PT-135	20	N	N	N	P	N	P	N
PT-136	ND	ND	ND	ND	ND	ND	ND	ND
PT-137	40	N	20	N	N	N	P	N
PT-138	40	N	20	N	N	N	N	N
PT-139	320	40	160	P	N	N	P	N
PT-140	40	N	N	N	N	N	N	N
PT-141	N	N	N	N	N	N	N	N
PT-142	N	N	N	N	N	N	N	N
PT-143	N	N	N	N	N	N	N	N
PT-144	N	N	N	N	P	N	N	N
PT-145	40	N	N	N	P	N	P	P
PT-146	20	N	N	N	P	N	P	P
PT-147	N	N	ND	N	N	N	N	N
PT-148	N	N	20	N	N	N	P	N
PV-25/92	320	320	N	N	N	N	N	N
PV49/92	160	640	N	N	N	N	N	N
SNV-123	160	80	N	N	N	N	N	N
SNV02/91	640	80	N	N	N	N	P	N
SNV07/89	20	20	N	N	N	N	N	N

APPENDIX 10:

CONTROL AREA: ARREPENDIDO

CODE	S	A	MH	SR	SL	HG	CQSE
ARR-001	M	10	Y1	N	N	0	0
ARR-002	F	8	N	N	N	750	0
ARR-003	F	5	N	N	N	450	0
ARR-004	F	3	N	N	N	ND	0
ARR-005	F	37	Y2	N	N	ND	0
ARR-006	M	4	N	N	N	310	2.2
ARR-007	M	11	N	N	N	1160	1.8
ARR-008	F	39	N	N	N	ND	6.5
ARR-009	M	4	N	N	N	730	10.5
ARR-010	M	7	N	N	N	ND	2
ARR-011	F	22	N	N	N	ND	1.5
ARR-012	F	21	N	N	N	ND	9.2
ARR-013	M	29	N	N	N	ND	1.5
ARR-014	F	15	Y1	N	N	ND	2.8
ARR-015	M	17	Y1	N	N	ND	1.5
ARR-016	F	15	N	N	N	ND	2
ARR-017	F	2	N	N	N	1210	1.6
ARR-018	M	11	N	N	N	ND	1.3
ARR-019	M	14	N	N	N	ND	3.6
ARR-020	F	34	N	N	N	ND	1.5
ARR-021	M	45	Y2	1	N	ND	1.5
ARR-022	M	4	N	N	N	1850	4
ARR-023	M	10	Y2	2	PV	>2600	1.5
ARR-024	M	7	N	N	PV	1465	1.5
ARR-025	M	5	N	1	PV	1235	2.1
ARR-026	M	4	N	N	N	ND	1.6
ARR-027	F	18	Y1	N	PV	ND	2.4
ARR-028	F	6	N	N	N	ND	2.1
ARR-029	F	10	N	N	N	ND	2.4
ARR-030	F	6	N	N	N	ND	6.5
ARR-031	F	13	N	N	N	ND	1.5
ARR-032	M	13	N	N	N	ND	4.2
ARR-033	F	10	N	N	N	1000	9
ARR-034	M	7	N	N	N	ND	180
ARR-035	M	5	N	N	N	ND	7
ARR-036	F	70	Y1	N	N	ND	1.5
ARR-037	F	54	Y1	N	N	ND	1.3
ARR-038	F	12	N	N	N	ND	1.3
ARR-039	F	11	N	N	N	ND	1.7
ARR-040	F	22	Y1	N	N	ND	2.3
ARR-041	M	36	YS	1	PV	ND	1.5
ARR-042	F	3	Y1	N	N	2560	1.3
ARR-043	F	2	N	N	N	ND	1.6
ARR-044	M	61	YS	N	N	ND	4.2
PF04/91	M	2	Y	1	PF	ND	ND
PF45/90	M	29	Y1	2	PF	ND	ND
PF53/89	M	29	Y4	N	PF	ND	ND
PF53/90	M	36	Y3	N	PF	ND	ND
PF54/92	M	12	YS	2	PF	<250	1.8

CODE	IF	IV	EB	CSF	CSV	CSM	CSK	CSN
ARR-001	N	N	N	N	N	N	N	N
ARR-002	N	N	N	N	N	N	N	N
ARR-003	N	N	N	N	N	N	N	N
ARR-004	N	N	N	N	N	N	N	N
ARR-005	20	N	N	N	N	N	N	N
ARR-006	N	N	N	N	N	N	N	N
ARR-007	N	20	N	N	N	N	N	N
ARR-008	20	N	N	P	N	N	N	N
ARR-009	N	N	N	P	P	P	P	P
ARR-010	N	N	N	N	N	N	P	P
ARR-011	N	N	N	P	N	N	N	N
ARR-012	N	N	N	N	N	N	N	N
ARR-013	N	N	N	N	N	N	N	N
ARR-014	N	20	N	N	N	N	N	N
ARR-015	N	N	N	N	N	N	N	N
ARR-016	N	N	N	N	N	N	N	N
ARR-017	N	20	N	N	N	N	N	N
ARR-018	N	N	N	N	N	N	N	P
ARR-019	N	N	N	N	N	N	N	N
ARR-020	N	N	N	P	N	P	P	P
ARR-021	20	N	N	P	P	N	P	P
ARR-022	N	N	N	N	N	P	N	N
ARR-023	N	N	N	N	N	P	N	P
ARR-024	N	20	N	N	N	N	N	N
ARR-025	N	N	N	N	N	N	N	N
ARR-026	N	N	N	N	N	N	N	N
ARR-027	N	20	N	N	N	N	N	N
ARR-028	N	N	N	P	N	N	N	N
ARR-029	N	N	N	N	N	N	N	N
ARR-030	N	N	N	N	N	N	N	N
ARR-031	N	N	N	N	N	N	N	P
ARR-032	N	20	N	N	N	N	N	N
ARR-033	N	N	N	P	N	N	N	P
ARR-034	N	N	N	P	P	N	P	P
ARR-035	20	N	N	N	N	P	N	N
ARR-036	20	N	N	N	N	N	N	N
ARR-037	20	N	N	N	N	N	N	N
ARR-038	N	N	N	N	N	N	N	N
ARR-039	N	N	N	N	N	N	N	N
ARR-040	N	N	N	N	N	N	N	N
ARR-041	20	20	N	N	N	N	N	N
ARR-042	N	N	N	P	N	N	N	N
ARR-043	N	N	N	N	N	N	N	N
ARR-044	320	40	20	P	P	P	N	P
PF04/91	160	320	N	N	N	N	N	N
PF45/90	80	80	20	N	N	N	N	N
PF53/89	320	N	20	N	N	N	N	N
PF53/90	640	80	N	N	N	N	N	N
PF54/92	640	N	N	P	N	P	N	N

APPENDIX 11

CONTROL AREA: INDIGENOUS AREA

CODE	S	A	MH	SR	SL	HG	CQSE
03/91	M	3	Y1	2	PF	0	1.5
04/91	M	48	Y	0	N	ND	2.1
05/91	M	41	Y	1	PF	ND	2.6
06/91	M	28	Y	0	N	ND	1.5
07/91	M	5	Y	0	N	2540	0
08/91	M	10	Y	0	N	1290	2.8
09/91	F	13	Y	0	N	715	4.9
10/91	F	34	Y	0	N	ND	4.1
11/91	M	4	Y	0	N	>2600	3.9
12/91	M	14	Y	0	N	920	4.1
13/91	M	21	Y	0	N	ND	1.3
14/91	F	31	Y	0	N	ND	0
15/91	M	32	YS	1	N	ND	0
16/91	M	34	N	O	N	ND	0
17/91	M	11	Y	1	PF	<250	1.5
18/91	F	12	Y	0	PF	0	2.1
19/91	M	4	Y	2	PF	<250	1.3
20/91	M	4	Y	1	PF	<250	1.7
21/91	M	2	Y	1	PF	<250	2.2
22/91	F	11	Y	2	PV	380	1.2
23/91	M	7	Y	0	N	770	1.3
24/91	F	1	Y	0	N	ND	1.5
26/91	M	21	Y	0	N	ND	0
27/91	M	21	Y	0	N	ND	0
PF15/91	M	4	Y	4	PF	0	ND
PF17/91	F	2	Y	3	PF	0	ND
PF45/92	F	32	Y4	0	PF	ND	0

CODE	EB	CSF	CSV	CSK	CSN	CSM	IF	IV
03/91	N	P	N	P	P	N	640	320
04/91	N	P	P	P	P	N	160	N
05/91	N	P	N	N	N	P	640	130
06/91	N	N	N	N	N	N	320	640
07/91	N	N	N	N	N	N	320	160
08/91	N	N	N	P	N	N	320	80
09/91	N	N	N	N	N	N	320	320
10/91	N	N	N	N	N	N	320	40
11/91	N	N	N	N	N	N	320	80
12/91	N	N	N	N	N	N	160	320
13/91	N	N	N	N	N	N	640	160
14/91	160	N	P	N	P	P	320	N
15/91	N	N	N	P	N	N	320	320
16/91	20	N	N	N	N	N	160	20
17/91	80	N	N	N	N	N	160	40
18/91	N	N	N	N	N	N	320	N
19/91	N	P	N	P	N	N	80	80
20/91	N	N	N	N	N	N	320	N
21/91	N	N	N	N	N	N	160	40
22/91	320	P	N	N	N	N	320	80
23/91	N	N	N	N	N	N	160	40
24/91	160	N	N	P	N	N	320	80
26/91	320	N	N	N	P	N	640	80
27/91	320	N	N	N	N	N	640	160
PF15/91	80	N	N	N	N	N	320	40
PF17/91	160	N	N	N	N	N	320	160
PF45/92	N	N	N	N	N	N	160	80

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PREVALENCE OF THE DIHYDROFOLATE REDUCTASE ASN-108 MUTATION AS THE BASIS FOR PYRIMETHAMINE-RESISTANT FALCIPARUM MALARIA IN THE BRAZILIAN AMAZON

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Abstract. Pyrimethamine resistance in cultivated laboratory isolates of *Plasmodium falciparum* is linked to the dihydrofolate reductase mutation Asn-108, a mutation that acts by interrupting drug binding within the active site of the enzyme. To determine the prevalence of this mutation in endemic regions harboring pyrimethamine-resistant malaria, we used a mutation-specific polymerase chain reaction assay to survey *P. falciparum* strains from a wide section of the Brazilian Amazon. Mutations were identified directly from blood samples without intervening steps of in vitro cultivation. Of 42 samples collected from four states in Brazil, 38 (90%) contained the Asn-108 codon AAC that confers pyrimethamine resistance, four samples contained only the wild-type Ser-108 codon AGC, and none contained the Thr-108 codon ACC found in cycloguanil-resistant pyrimethamine-sensitive strains. These findings indicate that a very high incidence of the Asn-108 DHFR mutation is responsible for pyrimethamine resistance in the Amazon, and they are consistent with recent failure rates reported for Fansidar (pyrimethamine-sulfadoxine). We suggest that limited use of proguanil be evaluated as an alternative to pyrimethamine.

Pyrimethamine and proguanil are antimalarial drugs that selectively inhibit *Plasmodium* dihydrofolate reductase (DHFR; EC 1.5.1.3, present in *Plasmodium* as a bifunctional enzyme with thymidylate synthase [EC 2.1.1.45]).[1] Since the introduction of pyrimethamine and proguanil nearly 40 years ago,[2,3] both drugs have been widely used in malaria prophylaxis and treatment. Unfortunately, drug-resistant *Plasmodium* has become widespread, and the use of pyrimethamine or proguanil (or cycloguanil, the active metabolite of proguanil) is no longer effective in many regions endemic for malaria.

Specific point mutations in DHFR confer differential resistance to pyrimethamine and cycloguanil in naturally resistant laboratory isolates of *P. falciparum*.[4,5] All pyrimethamine-resistant strains have been found to have a Ser-108 → Asn-108 mutation that produces only a small decrease in susceptibility to cycloguanil. In contrast, parasites with paired Ser-108 → Thr-108 and Ala-16 → Val-16 mutations have been found to be resistant to cycloguanil, but not to pyrimethamine. Mutations causing cross-resistance to both drugs have been reported for only two isolates from southeast Asia; both isolates exhibited the Ser-108 → Asn-108 mutation, a second Ile-164 → Leu-164 mutation, and a third Cys-59 → Arg-59 mutation.[4,5]

Since all pyrimethamine and cycloguanil-resistant isolates examined to date have point mutations in DHFR, resistance to these drugs may be predicted by assays that detect point mutations in the DHFR gene. Some methods of determining point mutations, such as restriction fragment length polymorphism analysis, cloning and sequencing, or hybridization with sequence-specific oligonucleotides are time consuming and labor intensive. Recently, however, methods have been described that use the polymerase chain reaction (PCR) for rapid and efficient detection of point mutations.[6–9] These methods are based on the observation that oligonucleotides having a single base mismatch at the 3′ end are much less efficiently extended during PCR than are perfectly matched primers. To detect point mutations responsible for pyrimethamine and cycloguanil resistance, we have developed a mutation-specific PCR assay that can be applied to a few drops of *P. falciparum*-infected blood. We report herein the results of a field study in which this assay was used to examine the incidence of DHFR Asn-108 among *P. falciparum* strains collected in the Brazilian Amazon.

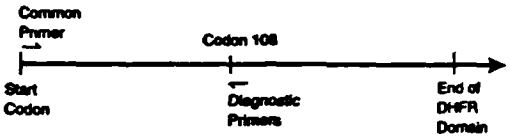

FIGURE 1. Strategy for PCR amplification of *Plasmodium falciparum* DHFR sequences in the mutation-specific assay. Positions of codon 108 and of sequences recognized by the PCR primers are indicated.

MATERIALS AND METHODS

Mutation-specific PCR assay

Three *P. falciparum* clones with known drug profiles were used to establish the mutation-specific PCR assay. Drug responses and DHFR sequences for each clone have been reported: [4, 10] the pyr^s-cyc^s 3D7 clone encodes Ser-108 (AGC), the pyr^s-cyc^r ItG2F6 clone encodes Thr-108 (ACC), and the pyr^r-cyc^s HB3 clone encodes Asn-108 (AAC). Parasite cultivation and DNA extraction were performed as previously described.[4]

Point mutations were detected by a mutation-specific PCR assay similar to that reported by Zolg and others[11] (Figure 1). Diagnostic primers DIA-3 5'-GAATGCTTTCCCAGC-3' (specific for Ser-108), DIA-9 5'-GAATGCTTTCCCAGG-3' (specific for Thr-108), and DIA-125'-GGAATGCTTTCCCAGT-3' (specific for Asn-108) were purchased from Synthecell Corporation (Rockville, MD). All diagnostic primers were used in conjunction with counterprimer SP15'-ATGATGGAACAAGTCTGCGAC-3'. PCR reactions were performed in standard reaction buffer[10] with 500 ng of a diagnostic primer and SP1. Forty-five cycles of amplification were completed, each consisting of denaturation for 30 sec (94°C), renaturation for 45 sec (56°C), and extension for 45 sec (74°C). Amplified products were stained with ethidium bromide and photographed after electrophoresis in agarose gels containing 1% standard agarose plus 2% NuSieve low melting-point agarose (FMC Bioproducts, Rockland, ME).

Blood samples and DNA preparation

Blood samples were collected before treatment from individuals who had not been under prior medical care for their malaria. Samples were directly cryopreserved[12] without in vitro cultivation. For DNA extraction, cells from 300 μl of each thawed sample were pelleted in a micro-

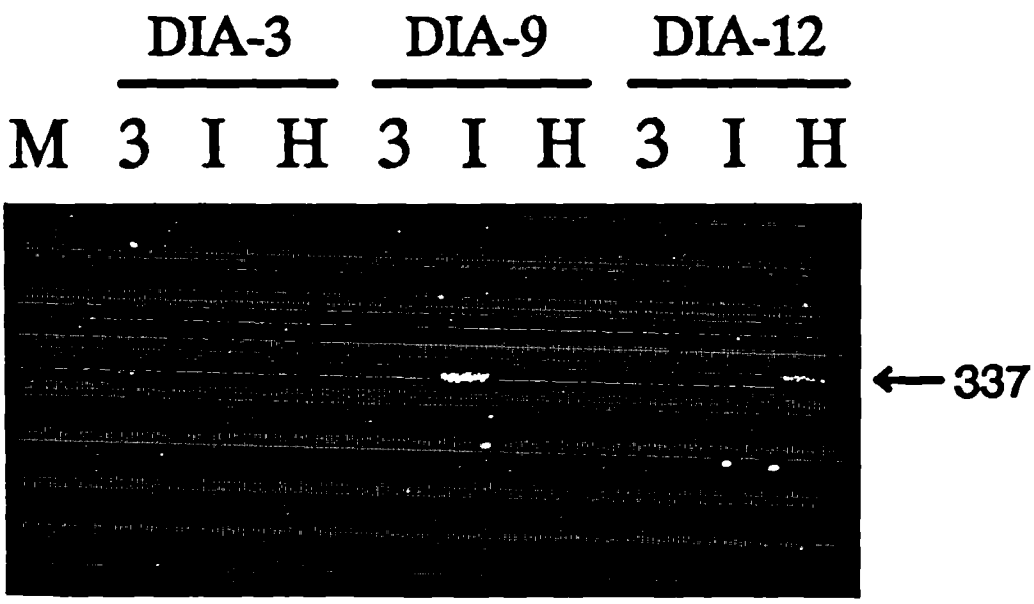

FIGURE 2. Mutation-specific amplification with diagnostic primers. DIA-3 detects the Ser-108 codon of the pyr^s-cyc^s 3D7 clone (lane 3), DIA-9 detects the Thr-108 codon of the cyc^r-pyr^s ItG2F6 clone (lane I), and DIA-12 detects the Asn-108 codon of the pyr^r-cyc^s HB3 clone (lane H). Each amplification was performed with counterprimer SP1 on 100 ng of genomic DNA for 32 PCR cycles. The arrow indicates the position of the 337-basepair PCR products detected by ethidium bromide staining after agarose gel electrophoresis.

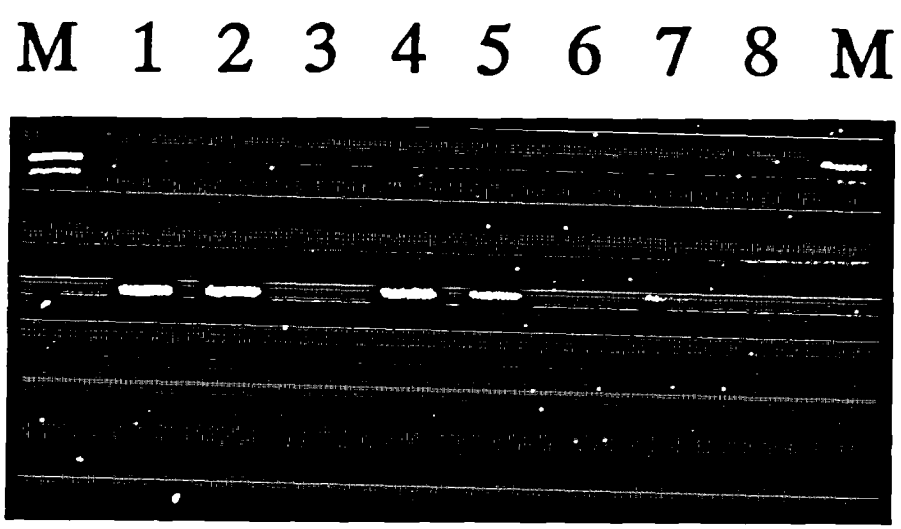

FIGURE 3. Sensitivity of the mutation-specific PCR assay. Lanes 1, 2, 4, 5, and 7 show amplification from samples containing 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001% parasite-infected erythrocytes, respectively. Lanes 3, 6, and 8 show that no amplified product was obtained from samples of unparasitized blood processed in parallel with parasitized samples. Lane M contains DNA size markers (ϕX174-*Hae*III plus λ-*Hind*III).

centrifuge, resuspended in 1,000 μl of TSE (100 mM NaCl, 20 mM Tris, 50 mM EDTA, pH 8.0), pelleted again, and resuspended in 1,000 μl of TSE containing 0.15% saponin. After incubation at room temperature for 2–10 min, parasites were recovered by centrifugation, resuspended in 500 μl of TSE and lysed by the addition of SDS and $NaClO_4$ to give final concentrations of 1% and 0.5 M, respectively. Samples were extracted twice with phenol:chloroform, once with chloroform, and precipitated with two volumes of ethanol. To monitor for cross contamination, a sample of unparasitized blood was processed in parallel with each sample of infected blood. Other precautions against contamination included processing of the blood samples and DNA preparations in a different part of the laboratory from that used to analyze the PCR reactions. Separate sets of pipettes were used for the analysis of the PCR products.

TABLE 1

Occurrence by state of Asn-108 and Ser-108 in the DHFR of Plasmodium falciparum *isolates*[*]

State	No samples	Ser-108	Thr-108	Asn-108	Mixed Asn-108 + Ser-108
Rondonia	21	2	0	18	1
Para	14	2	0	11	1
Mato Grosso	6	0	0	5	1
Amazonas	1	0	0	1	0
Total	42	4	0	35	3

[*] The sample points and years of collection were Costa Marques, Rondonia (1987, 1988, and 1989); Ariquemes, Rondonia (1987, 1988, and 1989); Porto Velho, Rondonia (1987, 1988, 1989, and 1990); Redenção, Para (1988); Itaituba, Para (1987, 1988, 1989, and 1990); Maraba, Para (1989); Belem, Para (1990); Guaranta do Norte, Mato Grosso (1988); Alta Floresta, Mato Grosso (1988); Peixoto de Azevedo, Mato Grosso (1988); Apuacas, Mato Grosso (1989); Camarua, Amazonas (1988).

RESULTS

The mutation-specific PCR assay is based on the observation that efficient amplification under stringent PCR conditions occurs only when there is a perfect match between the target DNA and the 3′ terminus of the diagnostic primer.[6–9] A single nucleotide change can thus be rapidly detected by a PCR primer having a 3′ terminal nucleotide complementary to the mutation. Detection of mutations at position 108 in *P. falciparum* DHFR is readily performed, since in all cases, Asn-108 has been found to be encoded by AAC, Ser-108 by AGC, and Thr-108 by ACC.[4, 5, 10, 13–15] Figure 2 shows that diagnostic primers having a 3′ terminus complementary to the second base of codon 108 produced clear discrimination of the Ser-108 (pyr^s-cyc^s), Thr-108 (cyc^t-pyr^t), and Asn-108 (pyr^s-cyc^s) mutations. A reannealing temperature of 55–56°C in the PCR cycle provided effective discrimination between mutations while providing high yields of amplified product. These findings are comparable to those of Zolg and others,[11] although

the assays differ in the primers used and the segments of the DHFR gene amplified.

The sensitivity of the mutation-specific PCR assay was assessed by combining known numbers of parasitized erythrocytes with 300 μl of uninfected whole blood. One-tenth of the DNA extracted from these samples was subjected to mutation-specific PCR amplification. Figure 3 shows that the limit of detection in these studies was better than 0.0001% parasitemia. Because of the extreme sensitivity of this technique, cross-contamination of samples was carefully avoided. In addition to standard measures to prevent contamination of the DNA extractions and PCR reactions, samples of unparasitized blood were included with the infected samples and processed in parallel during the assays. These precautions against contamination were effective; in all assays described, only a single instance of amplification was seen in a control reaction. In this case, analysis of the control and adjacent samples was repeated without reoccurrence of contamination.

Table 1 shows results from 42 samples collected during the years 1987–1990 from the states of Rondonia, Para, Mato Grosso, and Amazonas. Thirty-eight of the 42 isolates were found to have the DHFR Asn-108 mutation diagnostic of pyrimethamine resistance; three of these samples showed evidence of both Asn-108 and Ser-108. Since the erythrocyte stage of the *P. falciparum* parasite is haploid,[16] these three samples probably comprised mixed infections of pyrimethamine-resistant and pyrimethamine-sensitive parasites. The remaining four samples contained only the Ser-108 residue that is found in pyrimethamine-sensitive parasites. No sample showed evidence of the Thr-108 mutation that occurs in cycloguanil-resistant, pyrimethamine-sensitive *P. falciparum*.[4, 5]

DISCUSSION

Previous studies have demonstrated that the Asn-108 mutation in DHFR produces pyrimethamine resistance in *P. falciparum*. Patient isolates from pyrimethamine failures exhibit in vitro 50% inhibitory concentration (IC_{50} values) that are 500 to 1, 500-fold greater than IC_{50} values of susceptible isolates; these IC_{50} values have been linked to DHFR Asn-108 in a genetic cross.[4, 10] Biochemical studies have shown that this mutation interferes with the action of pyrimethamine by interrupting binding of the drug within the DHFR active site, an effect that is enhanced by the ancillary mutations Ile-51 and Arg-59.[10, 13, 14, 17] To date, parasite isolates from patients who have failed Fansidar treatment have all contained the DHFR Asn-108 mutation[10] (unpublished data). Other codons or alternative mechanisms of natural pyrimethamine resistance have not been reported.

In this study, we examined the incidence of the *P. falciparum* DHFR Asn-108 mutation across four states that span Brazil from the Bolivian border to the mouth of the Amazon River. Of these samples, 90% were found to harbor DHFR Asn-108, indicating that this mutation is the principal factor responsible for pyrimethamine-resistant malaria in endemic regions of South America.

The incidence of the Asn-108 mutation is comparable with recent failure rates reported for Fansidar (pyrimethamine-sulfadoxine). In vitro and in vivo drug response studies from the early 1980s documented 16–63% Fansidar failure rates from various regions of the Amazon.[18–21] More recently, with continued wide availability and use of the drug, 92% of samples collected from Acre state in 1987 were found to be Fansidar resistant.[22] This correlation between resistance and the incidence of Asn-108 suggests that potential augmentative effects of sulfadoxine in the Fansidar combination are inactive against the present strains of pyrimethamine-resistant *P. falciparum*.

Additional studies are needed to determine the cycloguanil responses and survey other DHFR mutations in the *P. falciparum* strains of South America. Clinical and field studies from other continents have found that proguanil (or the closely related analog chlorproguanil) can be effective against some strains of pyrimethamine-resistant falciparum malaria.[23–27] These findings can be explained by the different point mutations in DHFR that govern cycloguanil resistance.[4, 5]

Proguanil has been little used in South America.[28] Since Fansidar is now virtually ineffective on this continent, the use of proguanil on a limited basis may be of value. It is clear, however, that its wide use will lead to the spread of additional DHFR mutations and of proguanil-resistant malaria.

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Communication

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Wide Distribution of the Variant Form of the Human Malaria Parasite *Plasmodium vivax**

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We have found polymorphism in the repetitive and nonrepetitive regions of the sporozoite vaccine antigen, the circumsporozoite (CS) protein, in *Plasmodium vivax* malaria parasites from two geographically distant malaria endemic regions of the world. Like the recently described variant repeat sequence of *P. vivax* from Thailand, the CS protein repeat sequence of the variant *P. vivax* parasites from Papua New Guinea and Brazil is ANGA(G/D)(N/D)QPG, which differs from the previously identified CS repeat sequence, GDRA(D/A)GQPA, of *P. vivax* parasites from South America, Central America, and North Korea. Comparison of the *P. vivax* CS protein outside the repeat region revealed restricted polymorphism in regions that have exhibited T-cell immune function and sequence heterogeneity in the CS protein of *Plasmodium falciparum*. Our results show that *P. vivax* malaria parasites with the variant CS repeat sequences are widespread in nature and that the polymorphism in the CS protein of *P. vivax* is also present in the nonrepeat region.

The objective in the development of a vaccine against malaria, a disease that affects nearly half a billion people worldwide, is to intervene immunologically in the development of the parasite in the human host or *Anopheles* vector. Because malarial infections are initiated by sporozoites that are inoculated into a human by the bite of an infected mosquito, it has been proposed that the infection can be most effectively prevented by neutralizing the sporozoites (1). The CS[1] protein, which covers the surface of the sporozoite, is a candidate target for the production of a sporozoite-based malaria vaccine (1, 2). The gene encoding the CS antigen has been cloned from three of the four human malaria parasites (3–7). Immunologic experiments and sequence determination of the CS protein genes have revealed species-specific repetitive sequences (B-cell determinants) that make up the central one-third of the molecule and T-cell epitopes that reside in the nonrepeat part of the protein (8–10).

The nature and extent of variation in the vaccine-target immunodominant determinants of the CS protein of field-derived malaria parasites must be considered in the development of a subunit vaccine. Prior analyses of the CS proteins of *Plasmodium falciparum* parasites have revealed that polymorphism is restricted to the nonrepeat T-cell immunodominant regions of the molecule (11–13). Relatively less is known, however, about CS protein polymorphism in *Plasmodium vivax* parasites at the geographic level. In Thailand Rosenberg and his co-workers (14) recently fed mosquitoes on humans infected with *P. vivax*. These investigators isolated sporozoites from the infected mosquitoes and found that sporozoites from nearly 14% of the infected individuals had CS proteins with a variant repeat sequence, ANGAGNQPG. This repeat sequence, obtained from a clone designated as VK247, is different from the previously identified CS repeat sequence, GDRA(D/A)GQPA, of parasites from South America (Belem), El Salvador (Sal-1), and North Korea (NK) (5, 6, 15). Because the presence of variant forms of malaria parasite has implications for the efficacy of vaccine formulation, we have undertaken a study of polymorphism in the plasmodial vaccine candidate antigen from biologically representative field isolates of malaria parasites. The results presented here show widespread variant CS repeat- and nonrepeat-bearing *P. vivax* parasites in nature.

EXPERIMENTAL PROCEDURES

Isolation of Parasites—Field isolates of *P. vivax* employed in this study were from Paragaminos, Brazil and Madang, Papua New Guinea. Blood that had been microscopically confirmed as having *P. vivax* blood stage infection was collected for the isolation of genomic DNA.

Purification of the Parasite Genomic DNA and Amplification, Hybridization, and Sequencing of the CS Protein Gene—After the genomic DNA from field-derived parasites was isolated, 100 ng of the DNA was used in a polymerase chain reaction amplification of the CS gene using oligonucleotides AL 60 (GTCGGAATTCATGAAGAACTTCATTCTC) and AL 61 (CAGCGGATCCTTAATTGAATAATGCTAGG) as amplifying primers. These oligonucleotides, which correspond to the terminal regions of the CS gene, were designed to contain an *Eco*RI (AL 60) and a *Bam*HI (AL 61) site. Following 25 cycles of amplification, the amplified DNA was tested on gel, purified, and ligated into Bluescript plasmid DNA. The plasmid DNA was isolated from recombinant clones for hybridization and sequencing procedures. In preparation for hybridization experiments, oligonucleotides AL 114 (ATC AAC CAG GAG CAA ATG, complementary to a portion of the repeat sequence ANGAGNQPG), AL 116 (GGT GAT AGA GCA GAT GGA, complementary to a portion of the repeat sequence GDRAAGQPA), and AL 54 (CCA TGC AGT GTA ACC TGT GGA, complementary to the conserved region, Region II) were kinased. Three nitrocellulose slot blots con-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[TM] EMBL Data Bank with accession number(s) M69059-M69061

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[1] The abbreviations used are: CS, circumsporozoite; SDS, sodium dodecyl sulfate.

taining samples of the purified plasmid DNAs (10 per amplified DNA) were prepared. These blots were denatured, renatured, and baked before being prehybridized in 4 × SSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% SDS, and 0.4% bovine serum albumin at 42 °C for 30 min. The blots were hybridized at 42 °C overnight in the same buffer supplemented with tRNA. Posthybridization washes were carried out for 15 min in 6 × SSC, 0.1% SDS at room temperature followed by 5 min a wash in 6 × SSC, 0.1% SDS at 5 °C below the melting temperature of the oligonucleotides. The nucleotide sequence was determined using plasmid and CS gene-specific primers.

RESULTS AND DISCUSSION

Polymerase chain reaction-mediated amplification of *P. vivax* CS protein gene using genomic DNA from field isolates and *P. vivax* CS-specific primers produced a DNA fragment of about 1.2 kilobases. In a Southern blot experiment, these amplified fragments hybridized to oligonucleotides complementary to the conserved region, Region II of the CS protein gene (data not shown). The amplified CS DNA fragments from *P. vivax* parasites (14 from Madang and 30 from Paragaminos) were cloned into the plasmid vectors in preparation for hybridization and DNA sequencing. At least 10 recombinant clones from each transformation were analyzed by hybridization and/or nucleotide sequence analyses.

CS Repeat Sequence Polymorphism—In a hybridization experiment using oligonucleotides complementary to Region II and to both types of the CS repeats, we found that the CS gene clones from all *P. vivax* parasites hybridized to the Region II probe, but the hybridization was specific for either the ANGAGNQPG or the GDRAAGQPA repeat oligonucleotide probe (data not shown). Using this hybridization procedure with the 14 *P. vivax* isolates from Papua New Guinea, we found that one patient had parasites with a GDRAAGQPA CS repeat sequence, one had mixed infections with ANGAGNQPG and GDRAAGQPA repeat-bearing parasites, and the remainder of the patients were infected with parasites whose CS protein had the ANGAGNQPG repeat sequence. These findings differ from a previous serologic study that showed anti-GDRAAGQPA reactivity in a large number of serum samples from Madang (16). However, at the time the earlier study was conducted, the Thai type of the *P. vivax* CS repeat (ANGAGNQPG) had not been identified. Therefore, it would be useful to reevaluate the reactivity of those serum antibodies with the ANGAGNQP repeat peptides.

In the case of parasites from Brazil, however, a very different profile emerged from that found in Papua New Guinea. Of the 30 isolates analyzed, two had *P. vivax* with mixtures of ANGAGNQPG and GDRAAGQPA repeat-bearing parasites, and the remaining 28 isolates had parasites of the GDRA(D/A)GQPA CS repeat sequence. This within-isolate CS protein diversity underscores the need to analyze multiple recombinants from an individual transformation when characterizing parasite antigen genes. To determine the presence of parasite polymorphs within a patient, we sequenced 13 and 9 CS recombinants from isolates of P 19 and P 4 *P. vivax* parasites, respectively. Complete nucleotide and deduced amino acid sequences of four CS genes (P 19/D and P 4/B from Papua New Guinea, and B 7–4 and B 19–2 from Brazil) are presented in Fig. 1. Among the 13 CS clones sequenced from the P19 isolate 11 recombinants contained CS genes with the ANGAGNQPG repeat sequence, and two had the GDRAAGQPA CS repeat sequence. On the other hand, all nine CS clones sequenced from the P4 isolate had the ANGAGNQPG CS repeat sequence. These results were in agreement with the hybridization data.

We found 19 copies of the nonapeptide repeat sequence, ANGA(G/D)(N/D)QPG, in the CS gene of each of the parasites analyzed from Papua New Guinea and Brazil, compared with 18 units of repeats in the isolate from Thailand (VK247) (Fig. 1) (16). The repeat region sequence is polymorphic in the 5th (Asp/Gly) and 6th (Asp/Asn) residues. Even though glycine is present at positions 3, 5, and 9, and asparagine is present at positions 2 and 6 of the repeating unit, it is intriguing that the variation is oniy restricted to the 5th and 6th positions. This within-repeat polymorphism was not seen in the CS protein gene from Thailand. However, nonrandom third-base silent changes in the repeat region are shared by the CS gene of parasites from Brazil, Papua New Guinea, and Thailand, which probably indicates common origin and/or pressures (recombinational events) that maintain these repeat sequences.

Nonrepeat-based Polymorphism—Comparison of the nucleotide and deduced amino acid sequences outside the repeat region shows deletion/insertion and nonsynonymous changes (Figs. 1 and 2). Amino to the repeat region is a 24-nucleotide deletion in the P 4/B, B 7–4, and B 19–2 CS genes (Fig. 1). Like *P. falciparum* CS protein which has nonrepeat based variability, the CS protein of *P. vivax* is also polymorphic in regions outside the repeat domain. The three variable regions identified by our sequence comparison are amino to the repeat region, and amino and carboxyl to the conserved region, Region II. Amino to the conserved region, Region I, there is a nonsynonymous change from glycine to asparagine at the 38th amino acid position (Fig. 1). Another variable amino acid is post-Region II in a recently identified proliferative T-cell site, VTCGVGVRVRRRVNA(A/T)NKKP, of the CS protein in *P. vivax* (17) (Fig. 1). The third and the most variable region is amino to Region II. In this region, the amino acid residues that are polymorphic are 294, 295, 298, 310, 311, and 316 (Fig. 2). Also in this region, one CS clone, P 19/M, from isolate P 19 from Papua New Guinea had a deletion of 16 amino acids; the repeat sequences in this gene extend up to the polymorphic G/A position at amino acid 298. The CS gene of the previously identified North Korean *P. vivax* and a clone (P 19L) of the P 19 isolate from Papua New Guinea have an insertion of seven amino acids at position GNAGGNA, identified by an *asterisk* in Fig. 2. Sequence analyses of multiple clones of the CS protein gene from two isolates (P 19 and P 4) from Papua New Guinea revealed the presence of variants with polymorphic repeat and nonrepeat sequences (Fig. 2). Comparison of the CS protein gene sequences of six field isolates of *P. vivax* from distant geographic regions revealed that the variant amino acids in the nonrepeat region are shared.

Like the *P. falciparum* CS protein, the T-cell immunodominance of the *P. vivax* CS protein has not been extensively studied. Although more extensive studies to characterize the polymorphism in the CS protein gene of field isolates of *P. vivax* are needed to fully understand the nature and extent of antigenic variability, at least three facts seem to emerge from this study. First, the repeat-sequence polymorphism in *P. vivax* is similar to the already identified repeat-sequence variability in *Plasmodium cynomolgi* and *Plasmodium knowlesi* CS proteins (18, 19), which has not been seen in the *P. falciparum* CS protein. This difference in the nature of polymorphism between the sporozoite proteins of *P. falciparum* and *P. vivax*, *P. knowlesi* and *P. cynomolgi* is probably because the latter three parasites have evolved longer in response to host immune pressures compared with the recently evolved *P. falciparum* (20). However, this explanation may not hold true for blood-stage antigens, since mutations leading to selective advantages for the parasites are likely to be fixed rapidly in the blood forms, which are present in much greater numbers and exposed to the host immune system for a longer

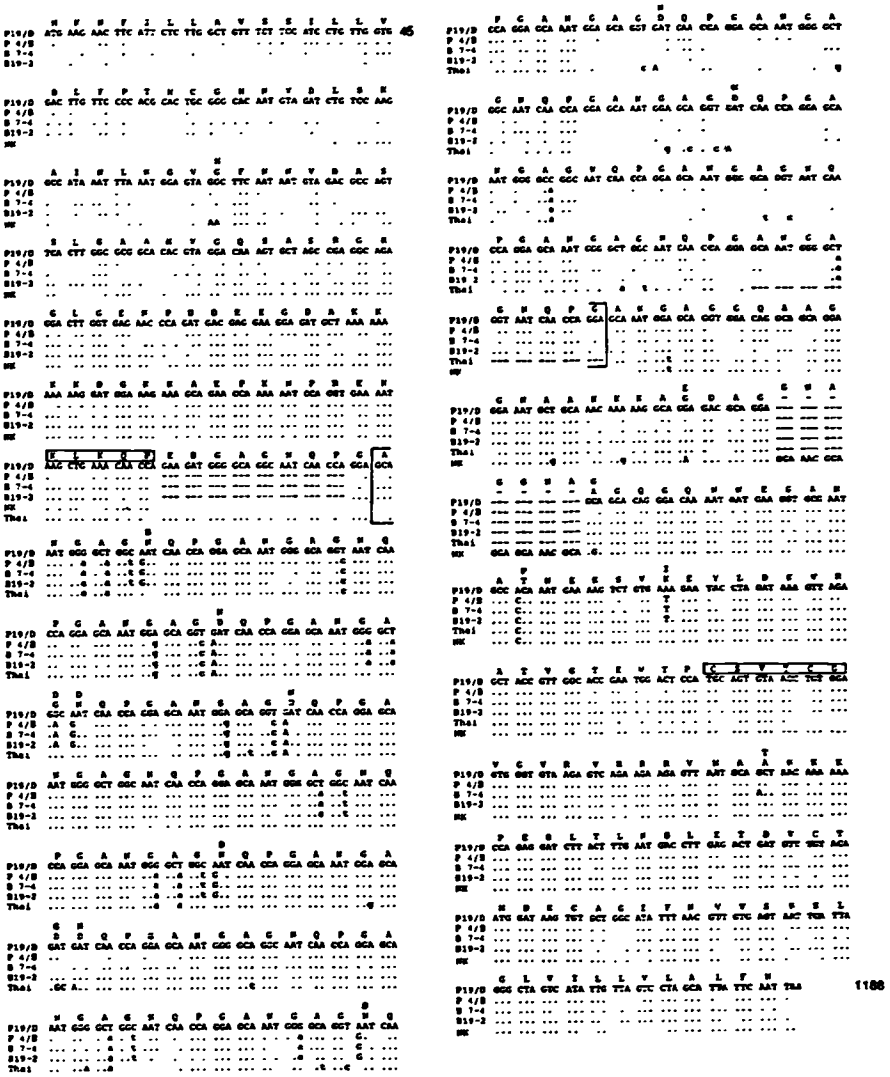

FIG. 1. **Nucleotide and deduced amino acid sequences of the CS protein of variant *P. vivax* parasites from Papua New Guinea and Brazil.** P 19/D and P 4/B are CS genes from parasites that originated from patients in Madang, Papua New Guinea. B 7-4 and B 19-2 are two CS genes from parasites isolated from patients in Paragaminos, Brazil. The CS gene sequence is compared with the published CS gene sequence of *P. vivax* (North Korean) in the nonrepeat region and with the CS gene of *P. vivax* (Thai, VK247) in the repeat region. A *period* indicates sequence similarity to the P 19/D sequence; a *dash* indicates deletions. Silent and nonsilent changes are shown by *lower* and *upper case letters*. Amino acid changes resulting from nonsilent nucleotide changes are shown.

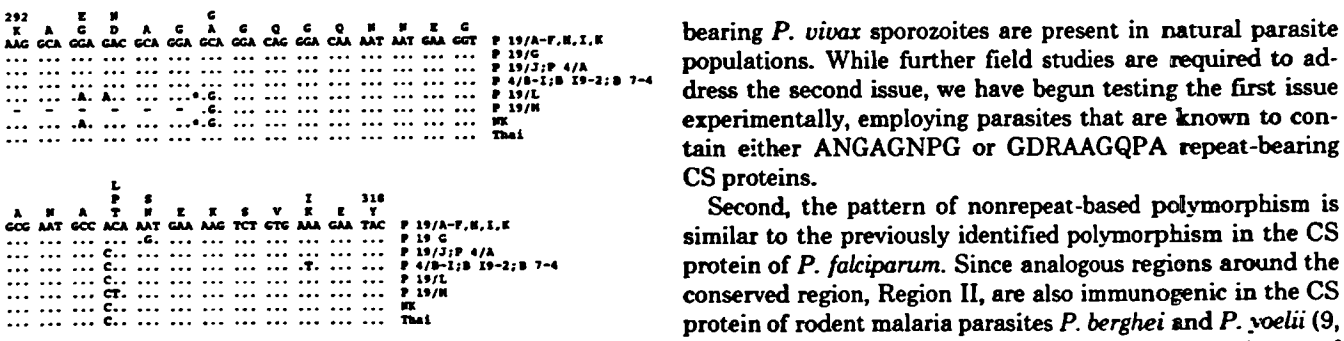

FIG. 2. **Comparison of the polymorphic pre-Region II CS protein sequence of *P. vivax*.** Amino acid residues in the polymorphic site (amino acids 292–318) are shown. The nonsilent nucleotide changes are shown by *upper case letters* underneath the nucleotide and deduced amino acid sequence of the representative sequence of P 19/A-F, H, I, and K. Previously characterized CS sequences of *P. vivax* CS proteins from North Korean (NK) and from Thailand (VK247) are also shown. The *dash* indicates a deletion in the CS protein gene in P 19/M. An *asterisk* in the NK and P 19/L sequence points to the insertion site of the seven-amino acid sequence GNAGGNA.

time than the sporozoite stage of the parasite. The obvious issues that emerge from this study are: 1) whether the CS repeat sequences exist as coding or noncoding cassettes in plasmodial genome, and whether genomic rearrangement results in generation of a variant repeat-bearing CS protein gene; and 2) whether more than two types of CS repeat-bearing *P. vivax* sporozoites are present in natural parasite populations. While further field studies are required to address the second issue, we have begun testing the first issue experimentally, employing parasites that are known to contain either ANGAGNPG or GDRAAGQPA repeat-bearing CS proteins.

Second, the pattern of nonrepeat-based polymorphism is similar to the previously identified polymorphism in the CS protein of *P. falciparum*. Since analogous regions around the conserved region, Region II, are also immunogenic in the CS protein of rodent malaria parasites *P. berghei* and *P. yoelii* (9, 21, 22), we speculate that the immunodominant epitopes of the *P. vivax* CS protein may reside in these polymorphic regions. Selective changes in the parasite protein sequences that interface with the host immune system can enable the parasites to evolve around host immune pressures, as has been suggested previously (23). In view of the above, one or more immunodominant determinants of the *P. vivax* CS protein are likely to reside in amino acids 292–318. Since the amino end of this polymorphic region (amino acids 292–318) is affected by insertion and deletion, unlike the rest of the region in this sequence, this part of the sequence may not contain immunologically and/or biologically relevant domains. However, this possibility needs to be tested experimentally.

Third, amino acid residues in the polymorphic positions are shared by CS proteins from distant geographic locations. This observation is similar to the finding for the *P. falciparum* CS

protein and reflects the extent of biologic and/or immunologic constraints on these regions and the independent origin of these changes. For vaccine considerations the development of "vaccine resistance" may be far less likely against a vaccine capable of inducing protective immunity with limited types of polymorphic components.

In summary, we have found that the variant form of *P. vivax* originally detected in Thailand is also prevalent in other malaria-endemic regions of Papua New Guinea and Brazil, though in different proportions. More importantly, we find that the repeat and nonrepeat polymorphic sequences of the *P. vivax* CS protein are shared by parasites from geographically distant regions of the world, thus indicating that a limited number of polymorphic parasites may have universal distribution. This limited universal polymorphism implies that a CS-based vaccine, alone or in combination with other antigens from sporozoite or other parasite stages, covering variant epitopes may be universally effective.

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Short Communication

Single amino acid variation in the ookinete vaccine antigen from field isolates of *Plasmodium falciparum*

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Pfs25, a 25-kDa protein expressed on the zygotes and ookinetes of *Plasmodium falciparum*, is a candidate vaccine antigen in the development of a transmission-blocking vaccine [1]. Monoclonal antibodies directed against the Pfs25 antigen inhibit parasite development in the mosquito midgut [2]. The gene encoding this protein has been cloned and sequenced from the human malaria parasite, *Plasmodium falciparum*; the avian malaria parasite, *Plasmodium gallinaceum*; and the chimpanzee malaria parasite, *Plasmodium reichenowi* [3–5]. Mouse antibodies against Pfs25 vaccinia virus recombinant vaccine have been recently shown to inhibit *P. falciparum* development in *Anopheles freeborni* mosquitoes [6].

In view of the polymorphic nature of other malaria parasite antigens, the sequence of the Pfs25 antigen from 8 laboratory cultured strains clones of *P. falciparum* was determined [7].The Pfs25 antigen of one parasite (clone Dd2) from Indochina has one non-silent substitution that leads to a conservative amino acid change (Ala to Gly) at the 132rd amino acid of the protein [7]. No silent nucleotide substitutions were seen in the 25-kDa antigen genes in that study. However, because the culture-derived parasites employed in the above study poorly infect mosquitoes, and because in vitro culture induces phenotypic and genotypic changes in parasites, we undertook a study of Pfs25 antigen variation employing field-derived *P.falciparum* parasites from Madang, Papua New Guinea (PNG), a holoendemic, high malaria transmission area, and Paragaminos, Brazil, a relatively low malaria-transmission area. Malaria parasites from 20 patients from Madang, PNG, and from 14 patients from Paragaminos, Brazil, were used in this study. The Pfs25 antigen gene of the parasites was amplified from the genomic DNA [8] using the oligonucleotide sequences GTCGGAATTCTTTTAAAAATGAATAAACTTTAC (AL80) and CAGCGGATCCTTACATTATAAAAAAGCATAC (AL81), which correspond to the two ends of the gene. The oligonucleotides were designed to carry the *Eco*RI (AL80) and *Bam*HI (AL81) restriction sites to facilitate cloning of the amplified fragments. Amplification of the genomic DNA yielded a 654-bp DNA fragment from all of the parasite DNA used in the study (data not shown). The DNA fragment was isolated and cloned, and one Pfs25 recombinant per isolate was sequenced.

We found that the Pfs25 antigen genes from all 14 isolates of *P. falciparum* from Brazil were identical to the previously described sequence from the 3D7 clone of the NF54 *P.falciparum* isolate [1]. On the other hand, sequence analyses of 20 isolates from PNG revealed

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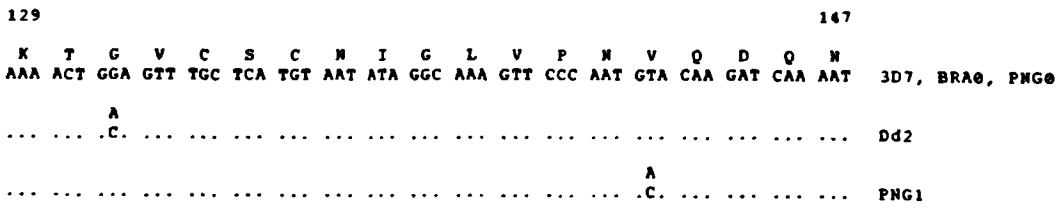

Fig 1. The nucleotide and the deduced amino acid sequence of the Pfs25 antigen gene is shown from amino acid 129 to 147. The sequences are presented in groups: 3D7, BRA0 (parasite numbers 4, 5, 6, 8, 9, 12, 15, 16, 17, 21, 22, 23, 24, and 26), PNG0 (parasite numbers 21, 27, 28, 41, 47, 52, 61, 65, and 72), and PNG1 (22, 31, 34, 38, 42, 45, 58, 68, 90, 92, and 98).

one non-silent and two silent changes in the Pfs25 antigen gene (Fig. 1). Six isolates had a silent nucleotide change at position 117 (T to C), and one isolate showed a silent nucleotide change at position 297 (T to C). The non-silent mutation at amino acid position 143 (GTA to GCA), found in 11 of the 20 isolates analyzed from PNG, resulted in a change from valine to alanine. This variable position is 12 amino acids carboxyl to the previously identified variable amino acid of this gene in the Dd2 strain of *P. falciparum* from Indochina [7]. This non-silent amino acid change, like the one observed previously, results in conservative amino acid substitution and is resident in a putative T-cell determinant of the molecule (Quakyi, I. A., personal communication). This limited and restricted nature of polymorphism in the Pfs25 antigen suggests that the antigen may not be expressed during the gametocyte stage in the human host, as was speculated earlier [1]. In such a case this antigen would not be exposed to host immune pressure, unlike the sporozoite and blood-stage antigens of malaria parasites. However, the vaccine-related relevance of the polymorphic amino acids needs to be determined in order to understand any vaccine-related implications of using Pf25 in a transmission- blocking vaccine.

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Polymorphism in the circumsporozoite protein of the human malaria parasite *Plasmodium vivax*

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The circumsporozoite (CS) protein that covers the surface of infectious sporozoites is a candidate antigen in malaria vaccine development. To determine the extent of B- and T-epitope polymorphism and to understand the mechanisms of antigenic variability, we have characterized the CS protein gene of *Plasmodium vivax* from field isolates representing geographically distant regions of Papua New Guinea (PNG) and Brazil. In the central repeat region of the CS protein, in addition to variation in the number of repeats, an array of mutations was observed which suggests that point mutations have led to the emergence of the variant CS repeat sequence ANGA(G D)(N D)QPG from GDRA(D/A)GQPA. Outside the repeat region of the protein, the nonsilent nucleotide substitutions of independent origin are localized in three domains of the protein that either harbor known T-cell determinants or are analogous to the *Plasmodium falciparum* immunodominant determinants, Th2R and Th3R. We have found that, with the exception of one CS clone sequence that was shared by one *P. vivax* isolate each from PNG and Brazil, the *P. vivax* CS protein types can be grouped into Papuan and Brazilian types. These results suggest that an in-depth study of parasite population dynamics is required before field trials for vaccine formulations based on polymorphic immunodominant determinants are conducted.

Key words: *Plasmodium vivax*; Circumsporozoite protein; Clonal typing; Epitope polymorphism

Introduction

In contrast to the progress towards vaccine development against *P. falciparum* malaria parasite, for which several stage-specific vaccine antigens have been characterized, studies of *P. vivax* parasite antigens have been limited, partly because of the lack of in vitro culture capability. Only two vaccine candidate antigens of *P. vivax* have been characterized: the circumsporozoite (CS) protein and the blood-stage antigen, PV 200 [1–7]. Immune responses against the CS protein have been studied, and vaccine formulations have been produced [8–16].

Vaccination with specific antigens or epitopes offers the possibility of inducing immunity against causative organisms of infectious diseases. However, natural polymorphism in the target determinants of the parasite proteins may compromise the efficacy of a subunit vaccine. Whether a vaccine is based on a critically important antigen present only in a single stage of the parasite or on a combination of important antigens present in several different stages, failure to deal with antigenic polymorphism could result in a vaccine that is unable to protect individuals exposed to a parasite with an antigenic variation. Therefore, whether the vaccine is targeted against single or multistage proteins, if a portion of a

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parasite population can escape the effects of a vaccine because of heritable polymorphism in vaccine target antigens. the natural population could rapidly evolve 'vaccine resistance' in a manner analogous to the development of drug resistance in malaria parasites. To ensure the efficacy of subunit vaccines based on polymorphic determinants. antigenic polymorphism of malaria parasites must be studied at the population level.

Polymorphism in the CS protein of *P. falciparum* has been shown to be restricted to the T-cell determinants [17–19]. However. polymorphism in the CS protein of *P. vivax*. which was originally observed in the repetitive B-cell determinant of the protein, has also been recently found outside the repeat region [5]. Variant *P. vivax* parasites from Thailand. Papua New Guinea (PNG) and Brazil have the repeat sequence ANGAGNQPG. which differs from the repetitive sequence GDRADGQPA of *P. vivax* CS proteins of parasites analyzed earlier [4,5]. We and others have subsequently shown that *P. vivax* parasites with the variant B-epitope repeat sequence have a wide geographic distribution [5,20]. In continuation of our earlier studies of the prevalence of CS proteins with the variant repeat sequence ANGAGNQPG in *P. vivax* field isolates from PNG and Brazil. we investigated: (1) nonsynonymous changes in the repeat region of *P. vivax* that result in the emergence of variant repeat sequences: (2) types of CS protein-bearing *P. vivax* parasites that predominate in PNG and Brazil; and (3) polymorphism outside the repeat region, in the T-cell determinants, of the *P. vivax* CS protein.

Materials and Methods

Origin of P. vivax parasites. Microscopically confirmed *P. vivax*-infected blood was collected directly from individuals living in a high malaria-endemic region of PNG (Madang) in March 1990 and from persons in relatively low malaria-endemic regions of Brazil (Paragaminos and around São Paulo) between 1986 and 1990.

Isolation and characterization of the circumsporozoite protein genes. As described earlier [5]. blood collected from patients infected with *P. vivax* was passed through a two-layer column of acid-washed glass beads and CF 11 cellulose to remove leukocytes. Parasite genomic DNA was isolated from infected erythrocytes, and 100 ng was used in a polymerase chain reaction (PCR)-mediated CS protein gene amplification. Oligonucleotides AL60 (GTCGGAATTCATGAAGAACTTCATTCTC) and AL61 (CAGCGGATCCTTAATTGAATAATGCTAGG) corresponding to the terminal ends of the CS protein were used as amplification primers. The PCR-generated fragments were digested with *Eco*RI and *Bam*HI. cloned in Bluescript plasmid. and transformed into *Escherichia coli*. The repeat sequence of the recombinant CS clones was identified by DNA:oligonucleotide duplex hybridization analysis using oligonucleotide probes AL114 (ATCAACCAGGAGCAAATG), and AL116 (GGTGATAGAGCAGATGGA) complementary to a portion of the repeat sequences ANGAGNQPG and GDRAAGQPA respectively. AL54 (CCATGCAGTGTAACCTGTGGA) complementary to the genus conserved CS region. R II was also used. CS gene-specific primers were used to determine the nucleotide sequence of the CS gene by the dideoxynucleotide method [21].

Results

On PCR-mediated amplification of the *P. vivax* CS protein gene amplification. genomic DNA extracted from blood samples of the patients from PNG and Brazil yielded DNA fragments approximately 1.2 kb in length. We sequenced the CS protein genes of a total of 115 CS clones representing 15 isolates from PNG and 24 from Brazil. The entire sequence of a total of 13 CS clones representing 8 *P. vivax* isolates from Brazil and 3 clones representing 2 isolates from PNG was determined. The CS genes of the remaining 102 *P. vivax* clones were sequenced to reveal sequences outside the repeat regions and portions of

the central repeat sequences. Of the 115 CS clones, we have previously described the complete sequence of 4 CS genes (P19 D. P4 B, B7 4, and B19/2) and the partial sequence of another 20 CS clones [5]. For the purpose of presentation. we are designating the GDRA(D/A)GQPA and ANGA(G D)(N D)QPG CS protein repeats as type 1 and type 2 repeats, respectively.

Within-isolate polymorphism of the circumsporozoite proteins. To determine the presence of parasite polymorphs among patients from PNG, we sequenced 13 clones from the P19 isolate, 10 clones from the P4 isolate, and 2 clones from each of the isolates P6, P7. P9. P10. P18, P25. P26. P72. and P73. A distinct variability in sequences was noted among the clones of P19. P4, P6, and P10 isolates; these variations ranged from a change of a single amino acid residue to insertion of a stretch of amino acids (Fig. 1). The isolate P19 was a mixture of both type 1 and type 2 CS repeat-bearing parasites; two clones, P19/L and P19 M. had type 1 repeat sequences, while the remaining 11 clones had type 2 repeat sequences, indicating that the variants coexist within a single isolate.

Similarly, polymorphism was noted in the repeat and nonrepeat regions of the *P. vivax* CS protein from Brazil (Fig. 1). We sequenced 8 clones from the B19 isolate, seven from the B37. 6 each from the B5 and B10 isolates, 5 from the B7, 3 each from the B11. B15, B21. B31. B34. B40, and B43, and 2 clones from the B14. B20, B26, B30. B38, and B39. Like the mixed infection observed in the P19 isolate from PNG, isolates B7 and B19 had both type 1 and type 2 repeat sequence bearing parasites. However, no amino acid variation was noted among 2–7 clones sequenced from each of the 11 isolates from Brazil.

Evolution of the repeat region of the circumsporozoite protein. Based on the nature of the non-synonymous changes, we have grouped the repeat domain of the CS genes of *P. vivax* into four categories (Fig. 2). The repeat sequences in rows 1–3 encode the type 1 CS repeat sequences, and the sequence in the

			RI				Repeats	RII											
Isolate	Clones	13	38	52	82			294	295	298	310	311	316	341	350	355	359	366	374	388
P19	9	L	G	V	A	*	Type 2	G	D	A	T	N	K	V	A	E	L	T	G	L
	1	.	.	.	.	*	.	.	.	.	.	S	.	.	.	.	.	.	.	.
	1	.	.	.	.	*	.	.	.	.	P	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	E	N	+G	P	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	–	–	G	L	.	.	.	.	.	.	.	.	.
P4	8	.	.	.	.		.	.	.	.	P	.	I	.	.	.	.	.	.	.
	1	.	.	.	.		.	.	.	.	P	.	.	.	.	.	.	.	.	.
	1	.	.	.	.	*	.	.	.	.	P	.	.	.	.	.	.	.	.	.
P6	1	.	.	.	.		.	.	.	.	P	.	I	.	.	.	.	A	.	.
	1	.	.	.	.		.	.	.	.	P	.	I	.	.	.	.	.	.	.
P10	1	.	.	L	.		.	.	.	.	P	.	I	.	.	.	.	.	.	.
	1	.	.	.	.		.	.	.	.	P	.	I	.	.	G	.	.	.	.
B7	4	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	.	.	.		.	.	.	.	P	.	I	.	T	.	.	.	.	.
B10	4	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	S
	1	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	S	.	.	.
B11	2	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	S	.
B15	2	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	–	–	G	P	.	.	A	.	.	.	.	.	.
B19	7	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	.	.	.		.	.	.	.	P	.	I	.	.	.	.	.	.	.
B21	1	.	N	.	V		Type 1	–	–	.	.	.	.	.	.	.	.	.	.	.
	1	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	.	.	.	.		Type 1	–	–	.	.	.	.	.	.	.	.	.	.	.
B40	2	.	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.
	1	P	N	.	.		Type 1	–	–	G	P	.	.	.	.	.	.	.	.	.

Fig 1. Parasite polymorphs within *Plasmodium vivax* isolates P designates isolates from Papua New Guinea and B from Brazil. ( ) indicates similarity with nine clones of isolate P19; ( ) indicates deletion. (+) EDGAGNQP amino acid residues insertion, and (–) GNAGGNA insertion (like NK strain, between residue number 297 and 298). The nucleotide and amino acid residue numbers correspond to the sequence P19 D reported by us earlier [5]

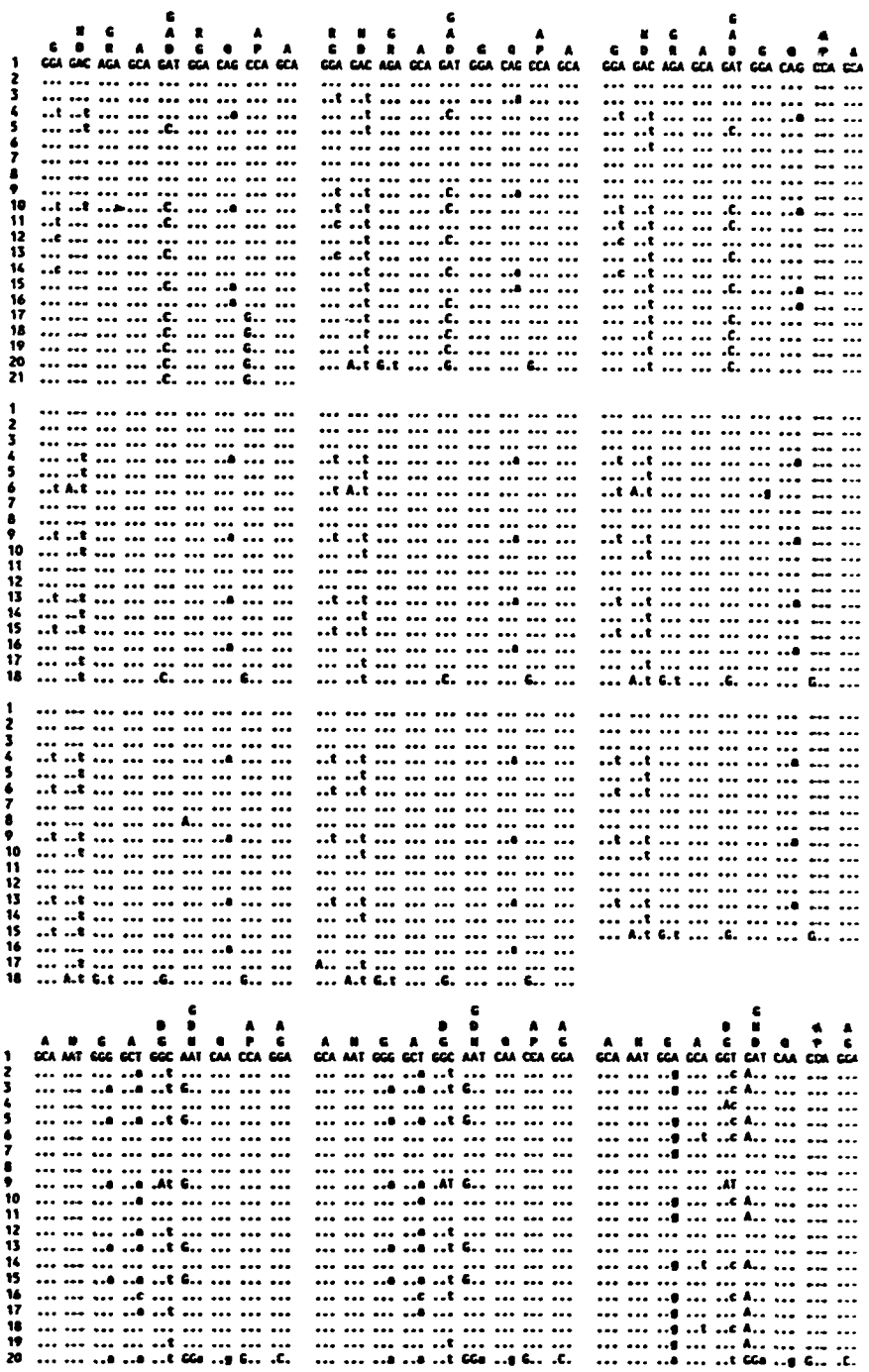

Fig. 2. Nucleotide sequence of the repetitive domains. Silent and non-silent mutations are shown by lower and upper case letters, respectively. Amino acid sequence and changes resulting from nonsilent nucleotides are shown in bold. Block 1: clones B7 5, B3 1 and B30 2 and B13 1; block 2: B5 1, B5 6 and B26 1; block 3: B38 2, B19 3 and B19 8 and B19 5; block 4: Repeat type-2 bearing clones P19 D, P19 B and P4 B, B7 4 and B19 2.

fourth row encodes the type 2 CS repeat sequence. Analysis of the nucleotide sequences of the diverging repeats reveals evolutionary relatedness between these sequences at both the nucleotide and amino acid level (Fig. 3). The CS protein repeat sequence from the parasites B7 5. B3 1, and B30 2, shown in the first row (Fig. 2), is similar to the previously identified sequence of parasites from Brazil [1]. El Salvador [2], and North Korea [3]. in that the 5th (D A) and 8th (P/A) amino acid residues in the repeating unit are polymorphic. One clone, B13 1, had no variation in the 8th residue. The CS protein repeat sequences of the B5 1, B5/6, and B26.1 clones, shown in the second row, differ from the above described set of sequences in that no polymorphism was found in the 5th or 8th residue in the repeat unit, except in the degenerative last repeat unit. However, polymorphism was noted in the 2nd amino acid position of the repeat (D N) in this group. The D/N transition was also found in the previous characterization of the CS gene from the North Korean isolate of *P. vivax*. but in the repeat units 4, 13. 14 and 20 [3]. The third group of the representative CS repeat sequences was seen in clones B38 2. B19 3, B19 8, and B19 5. which had no polymorphism except in the last degenerative repeat. In one clone, B38 2, there was a transversion from G to A at the 6th triplet codon of the 8th repeat.

The fourth category of repeat domain consists of the variant repeats identified in *P.*

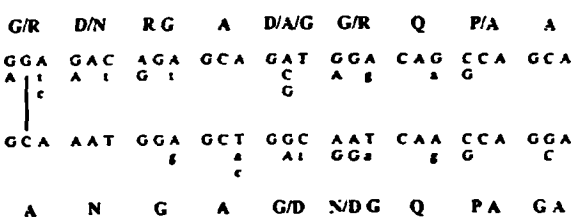

Fig. 3 Accumulation of silent (lower case) and nonsilent (upper case) mutations in a population of type-1 (top) and type-2 (bottom) repeat bearing clones. The deduced amino acid residues are shown in bold. A vertical line indicates the single point mutation that appears distinct in the differentiation of these two repeat types at the protein level

vivax parasites from PNG and Brazil. All clones from PNG (P19/D, P19/B, and P4 B) and from Brazil (B7 4 and B19/2) are polymorphic at the 5th and 6th amino acid residues, whereas the last degenerative repeat is polymorphic at the 8th and 9th positions as well. No polymorphism was noted in the repeat sequences reported from Thailand isolate [4].

Polymorphism in the nonrepeat region of CS protein. The comparison of the CS protein sequences outside the repeat region revealed that sequence polymorphism is restricted to three domains: amino to the conserved region. Region I (RI), and amino and carboxyl to the conserved region, Region II (RII) (Fig. 4). A 24-nucleotide insertion amino to the repeat region coding for EDGAGNQP was observed in 11 of the 13 clones in P19 and one of the 10 clones of P4 isolates from PNG. A similar insertion was originally seen in the CS gene of the VK247 *P. vivax* parasite [4].

Amino to the conserved region, RI, polymorphism is restricted to amino acid positions 11, 13, 38, 49, 52 and 82. We have found that CS proteins that bear type 1 CS repeats exhibit greater polymorphism in this domain. Among 73 type 1 CS clones analyzed, polymorphism was identified in 5 of 6 amino acid positions. In contrast, only one of 42 CS clones bearing type 2 repeats exhibited amino acid L instead of V at position 52.

The second and the most variable region is amino to RII. In this region, amino acid residues 294 (G E), 295 (D N), 298 (A/G), 310 (T P L), 311 (N S), and 316 (K I) are polymorphic in both type 1 and type 2 CS repeat-bearing parasites. The CS protein genes from PNG (clones P19 M and P22 C), which are similar to the CS protein gene from the North Korean (NK) strain [3], have a 16-amino-acid deletion 3' to the repeat sequence. This deletion is seen in all isolates from Brazil with type 1 repeats. The CS gene of PNG clones P19 L and P28 3 and the previously identified NK strain of *P. vivax* have a 7-amino acid insertion (GNAGGNA) at amino acid number 297 (Fig. 4). The *P. vivax* CS protein

	RI						Repeats	RII														
	11	13	38	49	52	82		294	295	298	310	311	316	341	350	355	359	364	374	388
1	S	L	G	A	V	A	* Type 2	G	D	A	T	N	K	V	A	E	L	T	G	L
2	.	.	.	.	.		* .	.	.	.	.	S	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	* .	.	.	.	P	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	.	.	.	.	P	.	I	.	.	.	.	.	.	.
6	.	.	.	.	L	.	.	.	.	.	P	.	I	.	.	.	.	.	.	.
7	.	.	.	.	.	.	.	.	.	.	P	.	I	.	T	.	.	.	.	.
8	.	.	.	.	.	.	.	.	.	.	P	.	I	.	.	G	.	.	.	.
9	.	.	.	.	.	.	.	.	.	.	P	.	I	.	.	.	.	A	.	.
10	.	.	N	P	.	.	Type 1	.	.	+G	P	.	.	.	.	.	.	.	.	.
11	.	.	N	.	.	.	Type 1	E	N	+G	P	.	.	.	.	.	.	.	.	.
12	.	.	N	.	.	.	Type 1	-	-	G	L	.	.	.	.	.	.	.	.	.
13	P	.	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	.	.	.	.
14	.	.	N	.	.	V	Type 1	-	-	G	P	.	.	.	.	.	.	.	.	.
15	.	.	N	.	.	.	Type 1	-	-	G	P	.	.	A	.	.	.	.	.	.
16	.	.	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	S	.	.	.
17	.	.	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	.	.	S	.
18	.	.	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	.	.	.	S
19	.	.	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	.	.	.	.
20	.	.	N	.	.	V	Type 1	-	-	.	.	.	.	.	.	.	.	.	.	.
21	.	.	.	.	.	.	Type 1	-	-	.	.	.	.	.	.	.	.	.	.	.
22	.	P	N	.	.	.	Type 1	-	-	G	P	.	.	.	.	.	.	.	.	.

Fig. 4. 'Clonal types' prevalent in Papua New Guinea (P) and Brazil (B). (•) and (−) are insertions EDGAGNQP and GNAGGNA respectively. 'Clonal-type': (1) 9 clones from single isolate (P19 A-F,H,I,K); (2) 1 clone (P19 G); (3) 1 clone (P19 J); (4) 3 clones from 2 isolates (P4/A, P25/C,D); (5) 24 clones from 11 isolates (P4 B-I, P3 C, P6 D, P7 C,D, P9 C,D, P15 C, P18 C,D, P26 C,D, P72 C,D, P72 C,D, P73 C,D, B19/2); (6) 1 clone (P10 C); (7) 1 clone (B7 4); (8) 1 clone (P10 D); (9) 1 clone (P6 C); (10) 1 clone (P28 C); (11) 1 clone (P19 L); (12) 1 clone (P19 M); (13) 1 clone (B13 1); (14) 1 clone (B3 1); (15) 1 clone (B15 3); (16) 1 clone (B10 3); (17) 1 clone (B11/2); (18) 1 clone (B10 2); (19) 61 clones from 23 isolates (P22 C, B4 1, B5 1-6, B7 2,3,5,6, B10 1,4-6, B11 1,3, B14 1,2, B15/1,2, B17'1, B19 1,4-9, B20 1,2, B21 2, B26 1,2, B28 1, B30 1,2, B31 1-3, B34'1-3, B37 1-7, B38 1,2, B39 1,2, B40 2,3, B42 1, B43/2,4); (20) 1 clone (B21 1); (21) 1 clone (B21 3); (22) 1 clone (B40 1).

sequence in this region, (T/P/L)(N/S)EKSV(K/I)EYLDKVRATVG, analogous to the T-helper site, Th2R, sequence of the *P. falciparum* CS protein, is also polymorphic. However, the immunogenic nature of this region in *P. vivax* needs to be established.

The third variable region is located carboxyl to the conserved region RII. In this region, polymorphism is restricted to amino acids 341 (V to A), 350 (A to T), 355 (E to G), 359 (L to S), 364 (T to A), 374 (G to S), and 388 (L to S). This polymorphic region contains the known T-cell determinants VTCG(V/A)GVRVRRR-VNA(A/T)NKKP, DLE(T A)DVA-TNDKA, and DKAA(G'S)IFNVVSN and a cell adhesion site VTCG(V A)GVRVR of the CS protein of *P. vivax* [9–11, 22]. In this region the *P. vivax* CS protein sequence (RVNA(A/T)NKKP(E G)DLT(L S)NDLE(T A), which is analogous to the CTL containing determinant, Th3R, in *P. falciparum*, is also polymorphic.

Clonal diversity of the circumsporozoite protein bearing parasites. To determine the proportional prevalence of parasites with polymorphic CS epitopes in two geographically distant malaria-endemic regions of the world, PNG and Brazil, we have grouped the CS protein sequences of 115 clones analyzed here, based on both the repeat region type and outside repeat region polymorphism. Our comparison identified 22 different 'clonal types' of CS proteins and revealed that with one exception in each PNG and Brazil, the CS clonal types of *P. vivax* parasites from PNG were distinct from those from Brazil. For instance, CS clonal type 5 was found predominantly in Madang, PNG; 24 CS clones representing 11 of 15 *P. vivax* isolates from Madang, PNG were clonal type 5. In contrast, clonal type 19 was most prevalent in *P. vivax* parasites from Paragaminos, Brazil; 61 clones representing 23 of the 24 isolates from Brazil contained this form of polymorphic CS protein.

Discussion

We have undertaken a longitudinal, population level study of the CS protein of *P. vivax* from two distant malaria-endemic regions, PNG and Brazil, to elucidate the nature and extent of epitope variability in this vaccine candidate antigen. We have found that both repeat and nonrepeat regions of the *P. vivax* CS protein are polymorphic. Sequence comparison of the repeat of the CS proteins, aligned in groups of type 1 and type 2 CS repeats, shows a pattern of both silent and nonsynonymous mutations and indicates that point mutations have led to the emergence of variant repeat sequences (Figs. 2 and 3). A single point mutation, G to C (second nucleotide of first triplet) in the common genetic pool of the CS clones analyzed here, representing type 1 and type 2 CS repeats, makes these two repeat types different at the protein level (Fig. 3). The array of mutations and amino acid substitutions present in the population of type 1 repeats suggests that this type of CS repeat sequence may have mutated to type 2 variant repeats: however, the reverse cannot be ruled out. Examination of repeat diversity in the CS proteins of other human malaria parasites showed a similar point mutation based diversity in the repeat sequence. For example, the change of the repeat sequence from NAAG to NDAG in *Plasmodium malariae* [23] and from NANP to NVDP in *P. falciparum* [24] can be explained by point mutations and subsequent expansion of the repeating unit.

Genetic recombination between homologous single-copy CS alleles during meiosis would not result in progeny with variant repeat types in the event of a single or even double crossover. However, mutations that contribute to the polymorphic nature of the regions outside the repeat region can be redistributed between the progeny. A new repeat type can only emerge if there is crossover inside the repeat region between alleles with different repeat types, in which case the progeny will have a part of each type of repeat with a distinct junction to predict the exact position of genetic exchanges. Such a genetic event, which has been seen in the *P. falciparum* merozoite surface antigen gene [25], has not been observed in CS protein genes. However, this does not rule out the existence of *P. vivax* parasites with CS repeat sequences distinct from the known type 1 and type 2 sequences.

In the nonrepeat regions of the CS protein, nonsilent nucleotide substitutions are localized in the identified T-cell epitope and in regions analogous to the immunodominant Th2R and Th3R regions of the *P. falciparum* CS protein. We propose that these two polymorphic regions may harbor immunodominant determinants of the protein; the underlying rationale is that mutations in the parasite's proteins that interface with host immunity, and which are advantageous to the parasite, would be positively selected rapidly [26]. This school of thought is contrary to the other opinion that favours DNA based selection and maintainance of polymorphism in the CS protein of human malaria parasites [27,28]. However, irrespective of the origin and the mechanism that maintains polymorphic sequence bearing parasites studies of antigenic polymorphism of parasite candidate vaccine antigens constitutes a step towards identifying vaccine-essential immunodominant determinants of parasite proteins. It remains to be determined experimentally whether Th2R and Th3R analogous regions in the *P. vivax* CS protein are immunogenic in nature. Information on the sequence variation of these regions would allow us and others to test the vaccine related effects of polymorphism.

Comparison of the CS protein gene sequences of field isolates of *P. vivax* from distant geographic regions, Brazil and PNG, revealed that each of these regions had distinct types of polymorphic CS protein-bearing parasites. However, we also found that certain types of polymorphic CS proteins were present in both PNG and Brazil. Moreover, mutations leading to individual amino acid changes were shared by Papuan and Brazilian parasites, indicating, as has been suggested before, a common but independent origin of sequence polymorphism

[29]. Our results suggest that both immunologic and genetic events are operational in the selection and expansion of the repeat sequence.

To summarize, we have shown that polymorphism in the CS protein of *P. vivax* is resident in both repeat and nonrepeat regions, that point mutations cause repeat sequence-based antigenic diversity in the CS protein, and that different clonal types of *P. vivax* predominate in the malaria-endemic regions of PNG and Brazil investigated here. Similar cross-sectional and longitudinal studies of vaccine antigen diversity are essential before malaria vaccine programs can be implemented in malaria-endemic regions. While a cross-sectional geographic level study would reveal the extent and proportional prevalence of polymorphic epitope-bearing malaria parasites, a longitudinal study at the population level would help clarify the dynamics of parasite populations as they relate to parasite infectivity and disease.

Acknowledgements

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DIVERSITY IN THE IMMUNODOMINANT DETERMINANTS OF THE CIRCUMSPOROZOITE PROTEIN OF *PLASMODIUM FALCIPARUM* PARASITES FROM MALARIA-ENDEMIC REGIONS OF PAPUA NEW GUINEA AND BRAZIL

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Abstract. To determine the nature and extent of variation in the T cell sites of the *Plasmodium falciparum* circumsporozoite (CS) protein, a candidate antigen in the development of a malaria vaccine, we cloned and sequenced 69 recombinant clones of the CS protein gene representing 18 and 17 *P. falciparum* isolates from infected individuals from Madang, Papua New Guinea (PNG), a holoendemic malaria region, and Paragaminos and Jacunda, Brazil, relatively low endemic regions, respectively. As previously known, the amino acid sequence polymorphism was restricted to the three immunodominant regions of the protein, Th1R-N1, Th2R, and Th3R. While some of the observed nonsilent mutations in the T cell determinants of the CS protein were similar to those previously identified, we have found new amino acid changes in each of the polymorphic sequences in parasites from PNG and Brazil. A comparison of the CS epitope sequences of parasites from PNG and Brazil with the previously known CS epitope sequences of parasites from Brazil and The Gambia showed the following: 1) polymorphism was found in the Th1R-N1, Th2R, and Th3R region; however, while amino acid substitutions in the Th1R-N1 and Th2R region tended to be conservative, the substitutions found in the Th3R region were not, suggesting that the Th3R epitope may be rapidly evolving to allow parasites to escape host antiparasite cytotoxic T cell-enforced immune responses, and 2) the CS proteins of *P. falciparum* from high malaria-transmission regions (PNG and The Gambia) appear more polymorphic than the CS proteins of parasites from relatively low malaria-endemic regions in Brazil, where *P. falciparum* infection has been recently established.

The worldwide spread of malaria and the number of people affected by this disease is growing at an alarming rate due to limited effectiveness of drugs and insecticides. In view of the failures of conventional malaria control measures, there is an urgent need for a malaria vaccine to help combat the disease. Several stage- and species-specific vaccine antigens of malaria parasite have been identified and are presently being evaluated for vaccine efficacy.[1] The circumsporozoite (CS) protein, which covers the surface of the infective sporozoite, is one of the candidate vaccine antigens.[2]

The immunodominant B cell epitope (repeat region) and T cell determinants of the CS protein of the human malaria parasite *Plasmodium falciparum* have been identified.[3–5] The T cell sites of the CS protein, Th1R-N1, Th2R, and Th3R, reside outside the repeat region of the protein.[5–9] Present efforts in sporozoite malaria vaccine development are directed towards inducing in vivo protective antibody and cytotoxic responses against the infecting parasite. For vaccine considerations, however, polymorphism in the immunodominant determinants in parasite populations can rapidly result in the selection of vaccine-resistant malaria parasites. In view of these considerations, several investigators have characterized the CS protein epitope sequences from laboratory and field *P. falciparum* parasites.[10–14] Sequence determination and hybridization analyses of the CS protein from laboratory-cultured isolates and sequence determination of the CS protein from field-derived parasites have shown various degrees of sequence polymorphism outside the repeat regions of the pro-

tein.[10 11] Immunologic experiments have shown that while some variant amino acids nullified the immune reactivity of the determinants, others did not effect the immune functions.[15-17]

We have undertaken a study of parasite surface protein variability in field isolates of parasites from low and high malaria-endemic regions. Our long-term goals are to identify the proportional prevalence of polymorphic determinant–bearing parasites in clinically nonimmune children and clinically immune adults in low and high malaria-endemic regions of the world. In the present study, we have characterized the CS protein genes of parasites from Madang, Papua New Guinea (PNG), a holoendemic *P. falciparum* malarious region, and Paragaminos and Jacunda, Brazil, relatively low malaria-endemic regions, and compared the polymorphic immunodominant sequences with available data on the CS epitope sequences of parasites from Brazil and The Gambia.

MATERIALS AND METHODS

Parasite origin and extraction of genomic DNA

Field isolates of *P. falciparum* used in this study were from two locations in Madang Province (Alexishafen and the town clinic), PNG and from Paragaminos and Jacunda Provinces in Brazil. *Plasmodium falciparum*–infected blood was obtained from persons with an age range of 2–36 years who lived either within a 40-km radius of Madang in PNG or in Paragaminos and Jacunda in Brazil. Diagnosis of the malaria parasite was done on site by microscopy. The majority of cases were pure *P. falciparum* or *P. vivax* infections; however, we found low-level infections of 1) *P. malariae* and *P. ovale* in PNG, 2) *P. malariae* in Brazil. and 3) dual *P. falciparum* and *P. vivax* infections in both Brazil and PNG. Following microscopic detection of *P. falciparum* infection, infected blood was collected by venipuncture into heparinized tubes and centrifuged. The plasma was separated and the buffy coat containing white blood cells was discarded. The parasitized red blood cells were frozen at −80°C until further use. Genomic DNA from 100 μl of infected blood was extracted in 0.5 ml of lysis buffer (50 mM Tris HCl, pH 7.5, 100 mM NaCl; 50 mM EDTA, pH 8.0, 1.0% sodium dodecyl sulfate) supplemented with 50 μg/ml of proteinase K. The extraction was carried out by incubation at 42°C for up to 1 hr. The genomic DNA was isolated from this lysis solution by consecutive extractions with phenol, phenol:chloroform, and chloroform. Following this organic extraction, the genomic DNA was precipitated in the presence of salt and ethanol and reconstituted in 200 μl of sterile water.

Amplification and sequencing of the CS protein gene

The CS protein gene was amplified from the genomic DNA (100–200 ng) using oligonucleotides AL 58 (GTCGGAATTCATGATGAG AAAATTAGCTATT) and AL 59 (CAGCG GATCCTAATTAAGGAACAAGAAGG), which are complementary and specific to the terminal ends (N and C terminals, respectively) of the CS gene of *P. falciparum*.[18]. These oligonucleotides were designed to contain an *Eco* RI (AL 58) and a *Bam* HI (AL59) restriction endonuclease cleavage site to facilitate cloning of the amplified DNA fragments into Bluescript (Stratagene, La Jolla, CA) cloning vectors. Following 25 cycles of amplification, the amplified DNA was analyzed on a 1.0% agarose gel. The amplified fragments (1.2–1.3 kb) were purified, digested with *Bam* HI and *Eco* RI, and ligated into appropriately digested Bluescript plasmid DNA. The recombinants were used to transform competent *Escherichia coli* (XL blue). Bacterial colonies containing the recombinant CS genes were identified, and plasmid DNA was isolated in preparation for nucleotide sequencing using plasmid and CS gene-specific primers.[19] An average of two clones per transformation were sequenced. The sequences of the primers used are AL 52, CAG GAA ACA GCT ATG AC; AL 53, GTA AAA CGA CGG CCA GT; AL 3, AAC ACA AGG GTT CTA AAT GAA TTA; AL 9, AAT AAA AAC AAT CAA GGT AAT; and AL 164, CAT GGG GAG GAT TCA GTT G.

RESULTS

The field-derived parasitized red blood cells were used in genomic DNA extraction to avoid any culture-induced artifacts. The amplification of the genomic DNA using CS gene–specific primers yielded 1.2–1.3-kb DNA fragments that hybridized to the conserved region, region II, and the repeat (NANP) sequence–specific oligonucleotide probe. We used the CS gene and plasmid

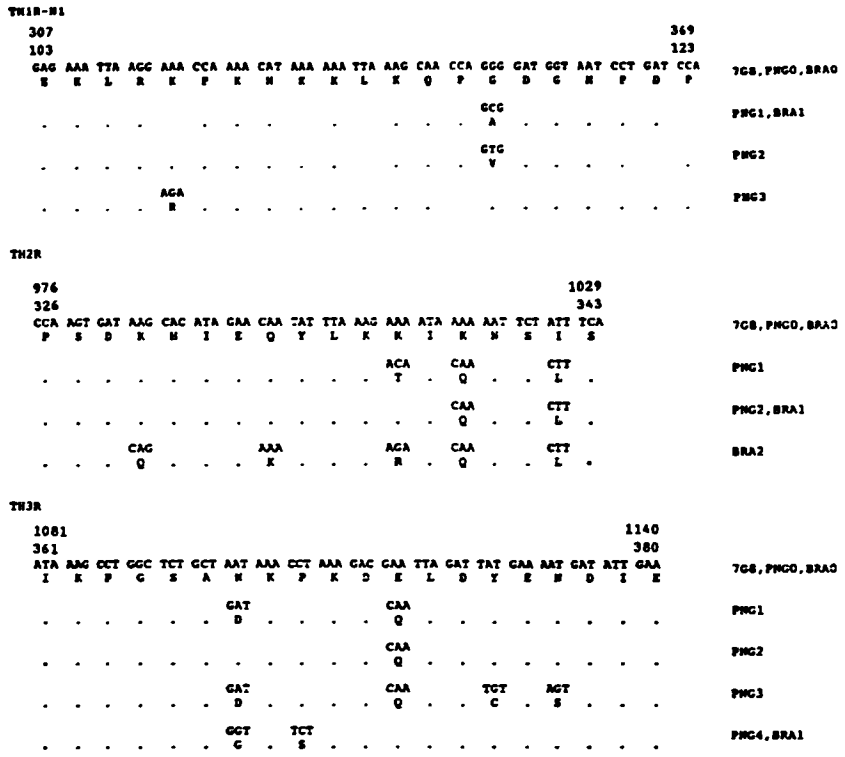

FIGURE 1. Variation in the three immunodominant determinants Th1R-N1, Th2R, and Th3R in the circumsporozoite (CS) protein gene of *Plasmodium falciparum* from Madang, Papua New Guinea (PNG), and Paragaminos and Jacunda, Brazil (BRA). Nucleotide and deduced amino acid sequences of the CS protein immunodominant determinants are compared with the sequences of a laboratory-cultured strain, 7G8, shown at the top. The sequences are grouped as PNG0-PNG4 for Papua New Guinea and BRA0-BRA2 for Brazil. The number represents the patient blood sample, and the letter denotes recombinant clones from an isolate. **Th1R-N1 region:** PNG0 (33 clones/16 isolates), 6B, 6D, and 6E; 20A, 20C, 20E, and 20H; 22B; 23E, 23B, and 23G; 24H; 27E; 31A and 31C; 38G; 45B and 45H; 52E and 52F; 56B, 56E, 56G, and 56H; 58E; 70E; 92A, 92B, 92C, 92F, and 92H; 93B; 102G. PNG1 (4 clones/3 isolates), 28E; 63I; 93G and 93H. PNG2 (1 clone/1 isolate), 58B. PNG3 (1 clone/1 isolate) 6F. BRA0 (19 clones/11 isolates), 1E; 2D; 5A; 7B and 7D; 8A and 8B; 9A and 9B; 13B and 13F; 14A and 14F; 16D and 16H; 21C and 21E; 26B and 26G. BRA1 (11 clones/7 isolates), 1A; 4A and 4B; 6A; 12A; 15A and 15C; 17A and 17B; 25A and 25B. **Th2R region:** PNG0 (3 clones/1 isolate), 23B, 23E and 23G. PNG1 (2 clones/2 isolates) 28E; 63I. PNG2 (34 clones/15 isolates), 6B, 6D, 6E, and 6F; 20A, 20C, 20E, and 20 H; 22B; 24H; 27E; 31A and 31C; 38G; 45B and 45H; 52E and 52F; 56B, 56E, 56G and 56H; 58B and 58E; 70E; 92A, 92B, 92C, 92F and 92H; 93B, 93G and 93H; 102G. BRA0 (19 clones/11 isolates), 1E; 2D; 5A; 7B and 7D; 8A and 8B; 9A and 9B; 13B and 13F; 14A and 14F; 16D and 16H; 21C and 21B; 26B and 26G. BRA1 (5 clones/4 isolates), 1A; 4A and 4B; 6A; 12A. BRA2 (6 clones/3 isolates), 15A and 15C; 17A and 17B; 25A and 25B. **Th3R region:** PNG0 (11 clones/5 isolates), 23E, 23B, and 23G; 45B and 45H; 56B, 56E, 56G, and 56H; 58E; 92C. PNG1 (21 clones/11 isolates) 6B, 6D, 6E, and 6F; 20A, 20C, 20E, and 20H; 22B; 24H; 38G; 52E and 52F; 58B; 70E; 92A, 92B, 92F, and 92H; 93B; 102G. PNG2 (2 clones/2 isolates) 28E; 63I. PNG3 (1 clone/1 isolate) 27E. PNG4 (4 clones 2 isolates) 31A and 31C; 93G and 93H. BRA0 (25 clones/14 isolates), 1E; 2D; 5A; 7B and 7D; 8A and 8B; 9A and 9B; 13B and 13F; 14A and 14F; 15A and 15C; 16D and 16H; 17A and 17B; 21C and 21E; 25A and 25B; 26B and 26G. BRA1 (5 clones/4 isolates), 1A; 4A and 4B; 6A; 12A.

DNA–sequence specific primers to determine the nucleotide sequence of 69 CS clones representing 18 and 17 isolates of *P. falciparum* from PNG and Brazil, respectively. The primers for sequencing of the CS gene were designed such that the entire outside repeat region and the ends of the repeat domain were sequenced. Figure 1 shows the nucleotide and the deduced amino acid sequences of the three polymorphic regions, Th1R-N1, Th2R, and Th3R. Based on the na-

ture of polymorphic sequences of the immunodominant determinants, we have categorized the sequences in groups PNG0 to PNG4 for parasites from PNG, and BRA0 to BRA2 for parasites from Brazil. The amino acid sequences of the polymorphic determinants are presented along with a comparison with the previously identified polymorphic sequences from The Gambia and Brazil in Figure 2.

In the region of the CS protein that contains the putative hepatocyte binding site and the proliferative T cell determinant composed of amino acids 103-123 (Th1R-N1), prior information on polymorphism is based only on the analyses of laboratory-derived parasites.[10] Of 18 isolates of *P. falciparum* from PNG and 17 isolates from Brazil used in the present study, the CS protein gene sequence of 33 clones representing 16 isolates in the PNG0 group and 19 clones representing 11 isolates in the BRA0 group was similar to the 7G8, Wel, and T9-101 CS genes.[3, 12, 20] The CS protein gene sequence of four clones representing three isolates in the PNG1 group and 11 clones representing seven isolates in the BRA1 group in the Th1R-N1 region was similar to the NF54, LE5, and T9-98 strains of *P. falciparum*.[10, 12, 13] Each remaining isolate from PNG had substitutions leading to amino acid changes not seen previously (PNG2 and PNG3). An overall comparison of the sequences shows that amino acid 117 (G/A/V) is most polymorphic in this domain of the CS protein.

Carboxyl to this variable region, in the alternating minor (NVDP) and major (NANP) repetitive sequence region, we found only one change: clone 23G of a PNG-derived isolate had NANS instead of NANP as the first repeating unit sequence, resulting from an initial base change of C to T. Two other CS clones (23E and 23B) from the same transformation had the NANP sequence as the first repeating unit. We do not know whether this observation represents a new repeat sequence of the CS protein in the process of emerging, such as the repeat sequence variation observed in *P. vivax*, *P. cynomolgi*, and *P. knowlesi* CS repeat sequences, or a degenerate repeat, as seen in *P. vivax* CS protein.[21-24] Further studies of field isolates are required to determine any repeat sequence–based polymorphism in the CS protein of *P. falciparum*.

Carboxyl to the repeat region, where the T cell immunodominant determinants (Th2R and Th3R) of the CS protein reside, we have found

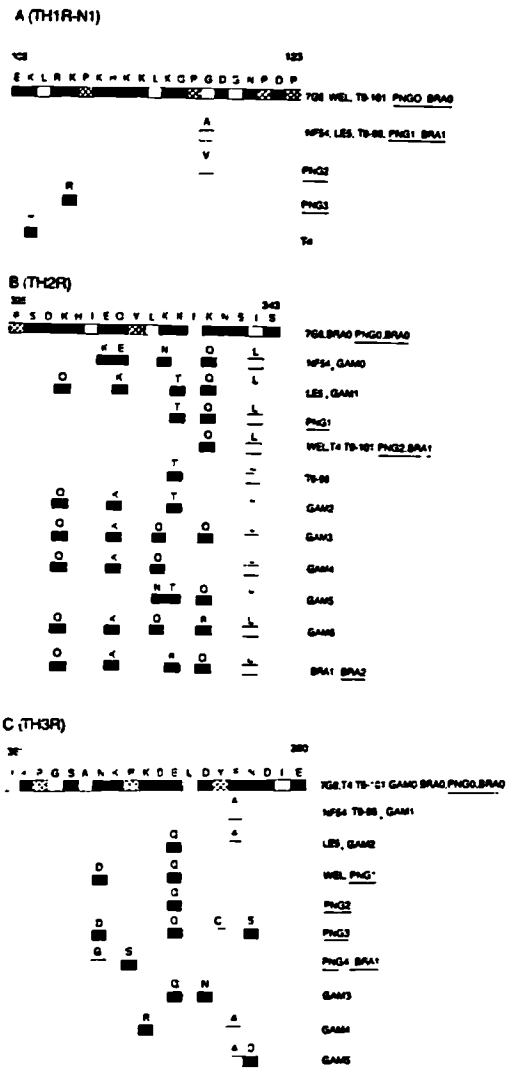

FIGURE 2. Hydrophilic and hydrophobic balance of the immunodominant epitopes of the circumsporozoite protein of field- and laboratory-derived *Plasmodium falciparum* parasites. A, TH1R-N1. B, TH2R. C, TH3R. The solid, open, and hatched boxes represent hydrophilic (usually on the protein surface), hydrophobic (usually internal), and neutral (both internal and external) amino acids, respectively. The geographic origin of the laboratory-cultured and field-derived *P. falciparum* isolates/strains are laboratory derived, 7G8 (Brazil).[3] LE5 (Liberia),[10] WEL (West Africa),[20] NF54 (The Netherlands; parasites were recovered from a traveler),[13] T4, T9-98, and T9-101 (Thailand);[12] field-derived, GAM0-GAM6 (The Gambia),[11] and BRA0-BRA2 (Brazil).[25] The description of the underlined parasite sequences from Papua New Guinea (PNG) and Brazil (BRA) is given in Figure 1.

a varying degree of polymorphism within the *P. falciparum* populations in Madang, PNG and in Paragaminos and Jacunda, Brazil. From 69 CS clones analyzed representing 18 isolates in PNG and 17 isolates in Brazil, variation in the Th2R could be grouped into four categories (Figure 1). Of 39 clones from PNG and 30 clones from Brazil, 34 from PNG (PNG2) and five from Brazil (BRA1) had sequences similar to the CS protein gene of Wel, T4, and T9-101 strains of *P. falciparum*. Nineteen CS clones representing 11 isolates from Brazil (BRA0) and three of the remaining five CS clones from PNG (PNG0) had Th2R sequences similar to the 7G8 CS sequence. Each of the remaining two clones (two isolates) of PNG had a sequence unlike any previously observed (PNG1). Six clones representing three isolates from Brazil (BRA2) had previously identified sequences.[25]

In the Th3R region of the CS protein, which contains a cytotoxic T cell determinant, we observed greater diversity than in the other two variable regions of the protein, Th1R-N1 and Th2R (Figure 1). In the Th3R region, the 39 clones from PNG can be grouped as follows: 21 (PNG1) were of the WEL type, 11 (PNG0) were of the 7G8 type, and the remaining seven (PNG2-4) represented five isolates that contained previously unknown sequences. Of the 30 CS clones from Brazil analyzed, only two types of variants were identified: 25 (BRA0) were of the 7G8 type, and the remaining five (BRA1) had sequences similar to PNG4.

Since a change in conformation due to amino acid changes can influence the immunogenicity of a determinant, we analyzed the changes in the hydrophilic amino acids (putative T cell receptor binding site [epitopic]) and in the hydrophobic amino acids (putative Ia binding site [agretopic]) of these polymorphic determinants.[6] In the Th1R-N1 and the Th2R regions, the observed polymorphism does not alter the hydrophobic and hydrophilic balance of the region (Figure 2A and B). In the case of Th2R, variation in all except one amino acid is resident in the putative T cell receptor domain of the amphiphilic complex, suggesting that the parasite is evolving in an epitopic rather than an agretopic fashion (Figure 2B). A very different picture emerged, however, when a similar comparison was made of the polymorphic sequences of the Th3R region (Figure 2C). Not only is this the most polymorphic region in parasite proteins from PNG and The Gambia, but the polymorphism in this region distinctly alters the amphiphilic balance of the determinant. Of the eight variable positions in this determinant, changes in four amino acids result in a change from a hydrophobic to a hydrophilic or a neutral amino acid, suggesting that this determinant, unlike Th2R, is evolving both in epitopic and agretopic fashions. Even though epitopic variation alone would aid parasites in evading immune surveillance, a combination of the epitopic and agretopic variation, as seen in the case of cytotoxic T lymphocyte (CTL)–containing TH3R, would increase the chances of the parasite to escape host immune surveillance. It is also possible that even a conservatively altered sequence may no longer be recognized in an individual primed with the original sequence.

To determine the nature and extent of the CS protein polymorphism in parasites from PNG. The Gambia, and Brazil, we compared the sequence of polymorphic determinants of the CS proteins (Figure 3). The results are presented as the percentage of variants bearing a polymorphic sequence in three malaria-endemic areas. In the Th1R-N1 region of parasites analyzed in this study, we found four polymorphic sequences in parasites from PNG and two polymorphic sequences in parasites from Brazil. Of the polymorphic sequences, the 7G8 type sequence was the most prevalent sequence in parasites from PNG (84.6%) and Brazil (63%). In the Th2R region, only four types of variants were found in *P. falciparum* parasites from PNG and Brazil (Figure 3) when compared with seven types of polymorphic sequences in parasites analyzed from The Gambia. Among these four types of variant Th2R sequences, the 7G8 type sequence was present in 71% of parasites from Brazil. whereas the variant type 3 Th2R sequence was present in 87% of parasites from PNG. When the polymorphism in the CTL-containing Th3R region was compared among three malaria-endemic areas, the results were different: only two types of variants were found in parasites analyzed from Brazil compared with five and six polymorphic Th3R epitopes. respectively, in parasites from PNG and The Gambia.

DISCUSSION

The polymorphic nature of malaria vaccine candidate antigens is of concern to vaccine developers. The CS protein gene sequence deter-

Sequence	GAM(%)	PNG(%)	BRA(%)
Th1R-N1:			
1) EKLRKPKHKKLKQPGDGNPDP		84.6	63.3
2) .....A.....		10.2	36.7
3) .....V.....		2.6	
4) ....R.....		2.6	
Th2R:			
1) PSDKHIEQYLKKIKNSIS		7.7	71.7
2) .....T.Q..L.		5.1	
3) .....Q..L.		87.2	9.4
4) ...Q...K...R.Q..L.			18.9
5) .....KE..N..Q..L.	18		
6) ...Q...K...T.Q..L.	2		
7) ...Q...K...T....L.	42		
8) ...Q...K..Q..Q..L.	4		
9) ...Q...K..Q.....L.	20		
10).....NT.Q..L.	2		
11)...Q...K..Q..R..L.	12		
Th3R:			
1) IKPGSANKPKDELDYENDIE	6	28.3	90.5
2) .....D....Q.....		53.8	
3) .....Q.....		5.1	
4) .....D....Q..C.S...		2.6	
5) .....G.S.....		10.3	9.4
6) .....A....	28		
7) .....Q...A....	30		
8) .....Q.N.....	30		
9) .....R.....A....	2		
10).....AD...	4		

FIGURE 3. Proportional prevalence rates of variants in the three immunodominant regions of the circumsporozoite (CS) protein from field isolates of *Plasmodium falciparum*. Variants of determinants in the CS protein of parasites from The Gambia (GAM) (50 clones/5 isolates),[11] Papua New Guinea (PNG) (39 clones/18 isolates), and Brazil (BRA) (53 clones/40 isolates; clones previously known[25] and our data combined) are compared. Polymorphic sequences of the CS protein of parasites are shown as percentages. The top sequence in each immunodominant region represents the laboratory strain 7G8. In the Th1R-N1 region, only variants from Papua New Guinea and Brazil are shown, since this region of the parasite proteins from The Gambia was not analyzed.

mination of a few laboratory-adapted *P. falciparum* clones and strains revealed that nonsynonymous nucleotide changes were localized in three T cell–recognizing regions.[10] Subsequent studies using field isolates have shown various degrees of polymorphism in the immunodominant determinants of the CS protein.[11, 25] There is a continuous debate on the origin, selection, and maintenance of polymorphism in the T cell immunodominant regions of the CS protein.[14, 26, 27] One hypothesis is that the human immune response is important in selection of variation, and another denies the existence of selectively maintained polymorphism in the regions of the protein that interface with host T cells. This is an important issue that needs to be explored to further our understanding of the genetic variability of malaria parasite antigens and the evolution of antiparasite host immune responses. From the perspective of vaccine developers, however, information about the nature and extent of epitope variation is crucial, regardless of how polymorphism in T cell sites arose.

Our comparison of polymorphic CS protein determinants from three distant malaria-endemic regions lends support to the hypothesis of host pressure–induced selection of polymorphic protein-bearing parasites. It appears that the primary function of the surface protein determinant(s) is to ensure successful host-parasite interaction. Nonsilent mutations in the regions of the CS protein that nullify parasite interaction with host cellular receptors will be rapidly selected against, and the representative parasites would disappear from the population of para-

sites. This would imply that the nonpolymorphic regions of the CS protein or a blood-stage antigen involved in host-parasite interaction may harbor the biologically sensitive targets of the protein.[28]

From an immunologic perspective, parasite protein sequences and/or conformations predicate the immune dominance or immunogenicity of a region, and the epitopes that interface with the host immune system would preferentially accumulate changes to escape the pressures of immunity. In situations in which biologically and immunologically sensitive regions of the protein coexist, accumulation of any nonsilent mutations would have to first pass through the biologic filter before being tested at the immunologic level. In the case of purely immunodominant determinants, however, nonsilent changes can be positively selected, particularly if the mutation results in an amino acid change that helps the parasite evade host immune pressures. In view of these considerations, the CTL-containing Th3R region would fit the definition of a true immunogenic site. It is only in this region that accumulated mutations result in altering of amphiphilic balance, as if the parasite is in the process of eliminating or changing the conformation of the CTL determinant.

Parasite protein polymorphism also needs to be examined from epidemiologic and entomologic perspectives. Our comparison of the prevalence of polymorphic amino acid sequences of the T cell epitopes shows that parasites in low malaria–endemic regions of Brazil are less polymorphic than parasites in high-malaria endemic regions of The Gambia and PNG. It remains to be determined whether the low-malaria endemicity of a region is due to presence of fewer polymorphic parasites, or to other human host and vector-associated factors. A longitudinal study of parasite protein polymorphism in low and high malaria–endemic regions would increase our ability to make intelligent guesses in this matter. The maintenance of parasite polymorphs is a function of the evolution of a population of parasites within a host and of a population of parasites within a population of hosts. We are continuing these studies of proportional prevalence of parasite polymorphs in different age groups in a longitudinal study in regions with both low- and high-endemic malaria to increase our knowledge of parasite mobility in different malaria-endemic regions of the world.

In conclusion, we have shown that the three T cell determinants of the CS protein of *P. falciparum* from PNG and Brazil contain new as well as previously identified polymorphic amino acid sequences. Our results suggest that the CTL-containing Th3R domain is evolving rapidly, as is evident from the nonconservative nature of its polymorphism compared with the conservative nature of polymorphism in the other two T cell determinants. We also found that the immunodominant determinants of the parasite are more conserved in a low malaria–endemic region than in two high malaria–endemic regions. These results and similar studies in the case of other vaccine antigens are essential in the development of malaria vaccines, and would help understand the complex immunologic, entomologic, and epidemiologic facets of malaria.

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